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The Role of Strigolactones in Lateral Root Development

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FREQUENTLY USED ABBREVIATIONS

ABC: ATP-binding cassette

ACC: 1-aminocyclopropane-1-carboxylate

AHK: ARABIDOPSIS HISTIDINE PROTEIN KINASE

ARF: AUXIN RESPONSE FACTOR

ARR: ARABIDOPSIS RESPONSE REGULATOR

ASA1: ANTHRANILATE SYNTHASE α1

CDD: CAROTENOID CLEAVAGE DIOXYGENASE

CK: cytokinin

CL: carlactone

COI1: CORONATINE INSENSITIVE1

Col-0: Columbia-0 accession

CTR1: CONSTITUTIVE TRIPLE RESPONSE1

D14: DWARF14

D53: DWARF53

EIN: ETHYLENE INSENSITIVE

ET: ethylene

ER: endoplasmic reticulum

ETO1: ethylene overproducer1

GFP: Green Fluorescent Protein

GUS: β-glucuronidase

IAA: indole-3-acetic acid

JA: jasmonate

JAZ: JASMONATE ZIM-DOMAIN

LR: lateral root

LRD: lateral root density

LRI: lateral root initiation

LRP: lateral root primordium

MAX: MORE AXILLARY GROWTH

MS: Murashige and Skoog basal salt mixture

NINJA: Novel Interactor of JAZ

NPA: 1-N-naphthylphthalamic acid

PAT: polar auxin transport

PIN: PIN-FORMED

qRT-PCR: quantitative reverse transcriptase polymerase chain reaction

RH: root hair

RSA: root system architecture

SCF: suppressor of kinetochore protein1 (SKP1), Cullin (CUL) and F-box protein**SHY2:** SHORT HYPOCOTYL2

SL: strigolactone

SMAX1: SUPPRESSOR OF MORE AXILLARY GROWTH2 1

SMXL: SMAX1-LIKE

TF: transcription factor

TIR1: TRANSPORT INHIBITOR RESPONSE1

Trp: tryptophan

WT: wild type

XPP: xylem pole pericycle

YUC: YUCCA

2,4-D: 2,4-dichlorophenoxy acetic acid

OBJECTIVES

The development and organization of the root system is pivotal for plant survival: it provides anchorage and assures the uptake of water and nutrients. Root development is influenced by nNumerous abiotic and biotic factors influence root development through the action of interacting phytohormonale homeostases and signaling pathways.

Strigolactones (SLs), firstly discovered for their role in the rhizosphere, also playhave an important role as plant hormones controlling various plant processes, of from which the regulation control of shoot branching has been studied in great detail. The action of a specific hormone is influenced by the spatio-temporally dependent interaction with other hormones and available metabolites. In the control of shoot branching, SLs act together with auxins and cytokinins in a tightly controlled network.

SLs have also been shown to be involved in shaping the root architecture, among others, by controlling lateral root development. Indeed, addition of SLs negatively influences the lateral root density, an effect that depends on the signaling component MORE AXILLARY GROWTH2 (MAX2).

The aim of this work is to get a detailed view on how SL signaling controls lateral root development and how it impinges on other known hormonal regulatory circuits that also control lateral root development.

The first objective of this thesis is to understand how SLs interact with cytokinins in the regulation of lateral root development. Cytokinins, just like SLs, negatively influence lateral root development. However, it is not well known whether both hormones interact. To reveal this interaction, spatiotemporal gene expression analyses were done together with genetic analyses.

The second objective of this thesis is to understand whether there is an interaction between SLs and ethylene and jasmonates, two stress hormones also known to control lateral root development, also by genetic and spatiotemporal gene expression analyses.

The third objective of this thesis is to elucidate which SL signaling components, other than MAX2, control the SL impact on lateral root development. The current hypothesis, based primarily on studies in *Arabidopsis* and rice (*Oryza sativa*), proposes that SLs are bound and hydrolyzed by the DWARF14 protein whereafter the SCF^{MAX2} complex ubiquitinates target

proteins for further signaling. Recently, DWARF53 in rice has been found to be a negative regulator of the SL effect on tillering. In *Arabidopsis*, a homologous protein has been detected: SUPPRESSOR OF MAX1 (SMAX1) that belongs to a small gene family. We wanted to investigate which members of the SMAX1 family are involved in the SL control of lateral root development.

In conclusion, these studies will provide a more detailed insight into how SLs, the most recently discovered plant hormones, regulate lateral root development.

Chapter I

Strigolactone biology and its crosstalk with other plant hormones

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Author contributions: LX.J wrote the chapter, B.M, C.M and S.G read and commented on the manuscript.

INTRODUCTION

Roots are essential for plant growth because they provide anchorage in the soil and allow water and nutrient uptake for efficient growth and development. The root system architecture (RSA) is defined as the shape and structure of the root system and consists of several aspects, such as main root length, number of lateral roots (LRs), LR length, adventitious root growth, and root angle (Hodge et al., 2009). Greatly depending on species, soil composition, and particularly availability of water and mineral nutrients, plants can optimize their root architecture by, for instance, initiating lateral root primordia (LRP) and influencing growth of primary roots or LRs. Root growth and branching result from the coordinated control of both endogenous genetic programs (growth regulation and organogenesis) and the action of abiotic and biotic environmental stimuli (Malamy, 2005; Lavenus et al., 2013; Jarzyniak and Jasiński, 2014; Lehmann et al., 2014; Grienenberger and Fletcher, 2015). In this process, several phytohormones and signaling pathways have a pivotal position.

Phytohormones are small molecules with a diverse chemical composition that are needed at very low concentration to carry out their function. Phytohormones are synthesized in defined organs and regulate cellular processes locally, but they are also transported to other parts of the plant to function at a distance from where they were produced. Five major groups of phytohormones have been defined: auxins, gibberellins, ethylene, cytokinins, and abscisic acid. In addition, other molecules have been found to regulate and influence plant growth and development and plant immunity, e.g brassinosteroids, jasmonates (JAs), salicylic acid, nitric oxide, signaling peptides, and more recently, strigolactones (SLs) (Raskin, 1992; Kauschmann et al., 2003; Reinbothe et al., 2009; Gomez-Roldan et al., 2008; Umehara et al., 2008; Hebelstrup et al., 2013).

Plant hormones influence and control plant growth by affecting gene expression patterns and usually by interacting with each other via their signaling pathways to regulate various mechanisms. In recent years, many studies have revealed cross-talk between hormones in different plant organs (De Rybel et al., 2014; reviewed by Vanstraelen and Benková, 2012; Pacifici et al., 2015). In this chapter, the effects and interactions between auxins, cytokinins, ethylene, JAs, and SLs in controlling root architecture will be discussed.

1. Lateral root development

The primary root of dicotyledonous plants can be divided in zones that depending on the cellular features are designated maturation zone, differentiation zone, elongation zone, and meristem zone. The latter is further divided into basal meristem zone, apical meristem zone and root cap (Fig 1). While LRs are formed in the elongation zone and emerged from the differentiation zone, they remain invisible, but are detectable to the naked eye in the maturation zone. Furthermore, roots are made up of specific tissues. The roots of the model plant *Arabidopsis thaliana*, which is the subject of this thesis, have a very simple histological structure with three external tissue layers, namely epidermis, cortex, and endodermis, surrounding a single-layered pericycle and the inner vasculature (Dolan et al., 1993) (Fig 1).

LRs are a major characteristic of the RSA. They extend the ability of the root system of the plant to acquire nutrients and water from the substrate in which they grow. In *Arabidopsis*, LRs originate exclusively from the pericycle cells that are located opposite to the xylem poles (Dolan et al., 1993; Péret et al., 2009). In general, the LR formation process can be divided into three major steps defined as priming, initiation, and emergence (Péret et al., 2009; Lavenus et al., 2013). Priming of the xylem pole pericycle (XPP) cells takes place in the basal meristem zone,

which is nearest to the root tip, together with auxin oscillation responses in the root (De Smet et al., 2007). As the root grows, the primed XPP cells enter the elongation zone, where the first asymmetric cell division of the founder cells occurs, leading to the LRP formation (Dubrovsky et al., 2000, 2001; Lavenus et al., 2013). Then, the subsequently controlled cell divisions give rise to a typical dome-shaped primordium and, finally, an emerged LR (Malamy and Benfey, 1997; Lavenus et al., 2013). An overview of the *Arabidopsis* LR development will be given by using these three steps.

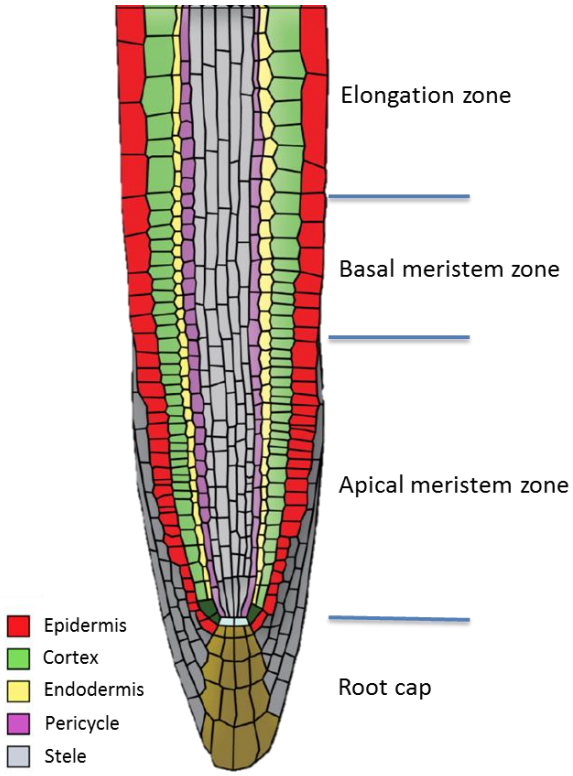

Figure 1. Structure and different cell types in the *Arabidopsis* root. The *Arabidopsis* root has a simple structure. It is composed of epidermis, cortex, endodermis, pericycle, and vasculature. Each tissue possesses a single cell layer. Based on the cell activities, the primary root tip can be divided into an elongation zone, basal meristem zone, and apical meristem zone. In the LR formation process, priming

of xylem pole pericycle (XPP) cells and lateral root initiation (LRI) take place in the basal meristem zone and the elongation zone, respectively. The figure has been adapted from Péret et al., 2009.

1.1 Priming of the XPP cells

In *Arabidopsis*, the pericycle is a heterogeneous cell layer consisting of two different cell types (Dolan et al., 1993, Parizot et al., 2008): cells located in front of the two phloem poles that display features of differentiated cells and XPP cells located opposite xylem poles that show a dense cytoplasm and a fragmented vacuole that are characteristic of meristematic cells (Parizot et al., 2008; Dubrovsky et al., 2008). In addition, XPP cells are shorter than the other pericycle cells.

The specification of these different pericycle cells has been suggested to occur in the initiation of the stele and to correlate with the vascular root organization (Parizot et al., 2008), as illustrated, for instance, by the recessive mutant *lonesome highway (lhw)* that shows loss of diarch organization of the vasculature and also a specification block of the pericycle cells (Ohashi-Ito and Bergmann, 2007). Another example is the phenotype of the *wooden leg (wol)* mutant in which the vascular heterogeneity is suppressed and the heterogeneity in the pericycle is interrupted (Parizot et al., 2008).

However, not every XPP cell will create an LR, because LRI takes place in a spatially controlled manner (Van Norman et al., 2014). Although cell division is required for the formation of the multicellular primordia, triggering of the cell cycle in the pericycle cells is not sufficient by itself to induce the primordium initiation (Vanneste et al., 2005; De Smet et al., 2010). Growth regulators, such as phytohormones, especially auxin, are reported to play an important role in

this case. For instance, the gain-of-function mutation in *SOLITARY ROOT* (*SLR*)/*INDOLE-3-ACETIC ACID INDUCIBLE14* (*IAA14*), in which the early auxin response is blocked, displays no LRI (Fukaki et al., 2002). 1-*N*-naphthylphthalamic acid (NPA) is a negative regulator of polar auxin transport (PAT) and its application prevents the progression of the pericycle cells through the cell cycle (Himanen et al., 2002; Vanneste et al., 2005). This defect can be overcome by adding auxin to the plants (De Smet et al., 2010). Recurrent local accumulation of auxin in the protoxylem cells from the basal meristem has been linked to the priming of the XPP cells (De Smet et al., 2007). As a result, priming is defined as a cyclic pre-initiation event, governed by a cyclic auxin peak, through which XPP cells go to become prebranch sites that have the potential to initiate an LR that become LR founder cells (Lavenus et al., 2013).

Concerning the auxin signaling pathway, the IAA28-dependent auxin signaling has been suggested to be required for priming LR founder cells (Rogg et al., 2001; De Rybel et al., 2010; Lavenus et al., 2013). Other mutants of the auxin influx transporter (AUX)/IAA family members, such as *iaa8-1* and and the IAA19 loss-of-function mutant *massugu2* (*msg2*), are also defective in LR formation (Tatematsu et al., 2004; Arase et al., 2012). *IAA8* and *IAA19* were shown to be expressed in the basal meristem zone (Groover et al., 2003), indicating that these two genes might play a redundant role in priming founder cells together with IAA28 (Dreher et al., 2006). Downstream of the AUX/IAA proteins, the AUXIN RESPONSE FACTOR (ARF) transcription factors control the auxin-dependent gene expression. Several ARFs have been found to interact directly with IAA28 and IAA8 to prime XPP cells (De Smet et al., 2010; De Rybel et al., 2010; Arase et al., 2012). Yeast two-hybrid analyses revealed that IAA28 interacts with ARF5, ARF6, ARF7, ARF8, and ARF19 (De Rybel et al., 2010) and IAA8 with ARF5, ARF7, and ARF19 (Arase et al., 2012). Together, these results suggest that IAA28 (and IAA8 and IAA19 as well)-ARFs (ARF5, ARF6, ARF7, ARF8, and ARF19) are important auxin-

dependent signaling modules involved in priming of XPP cells. Very recently, the indole-3-butyric acid (IBA)-derived IAA in the root cap has been found to play an essential role in priming XPP cells and the MEMBRANE-ASSOCIATED KINASE REGULATOR4 (MAKR4) has been proposed to function downstream of the IBA-to-IAA converstion pathway to convert a prebranch site into an LR (Xuan et al., 2015).

Besides auxin, other phytohormones are also involved in priming of XPP cells. Recently, carotenoid biosynthesis has been suggested to function in the earliest steps of LR development before the development of primordia (Van Norman et al., 2014). Disruption of the carotenoid biosynthesis resulted in seedlings with few LRs and closer examination indicated that this biosynthesis is required for prebranch site formation. Carotenoids are considered as precursors of abscisic acid and SLs, both shown to regulate auxin fluxes during LRI (Seo and Park, 2009; Guo et al., 2012; Thole et al., 2014; Ruyter-Spira et al., 2011; Koren et al., 2013). However, pharmacological studies as well as mutant analysis indicated that neither abscisic acid nor SL deficiency was the cause of the phenotype (Van Norman et al., 2014). Hence, an uncharacterized apocarotenoid has been proposed to be implicated in this early LRI step (Van Norman et al., 2014).

The primed XPP cells are referred to as founder cells. A founder cell is the first cell initial that is destined to become an organ or a cell type (Chandler, 2011). Although understanding of the signaling pathway that regulate the formation of founder cells is being unraveled, the molecular components for the founder cell specification process are still lacking. GATA23 is a transcription factor suggested to be a candidate to control the founder cell specification process because the expression of *GATA23* correlates with oscillating auxin signaling maxima in the basal meristem zone and occurs specifically in XPP cells before the first asymmetric division

(De Rybel et al., 2010; Yadav et al., 2010; Singh et al., 2012). Moreover, the expression of *GATA23* depends on the IAA28-ARF7/ARF19 module mentioned above (De Rybel et al., 2010).

1.2 Lateral root initiation

After pericycle cells have been specified, they move upward from the root apical meristem zone to the basal meristem zone. Pericycle cells opposite the xylem poles proceed to the G2 phase preparing to receive a signal to divide, whereas the other pericyle cells reach their maturation and remain in the G1 phase (Dubrovsky et al., 2000; Beeckman et al., 2001), indicating that the mitotic competency of XPP cells is crucial for LRI. The mutant *aberrant lateral root formation4 (alf4)* blocks the expression of B1-type cyclin *CycB1:GUS* (a G2-to-M phase marker of the cell cycle), but causes the overexpression of the earlier B-type cyclin-dependent kinase *CDKB2:GUS* (an early G2-to-M phase marker) in the XPP cells (DiDonato et al., 2004), implying that the ABERRANT LATERAL ROOT FORMATION4 (ALF4) protein is required to keep XPP cells in a mitosis-competent state. Recently, the cell cycle regulator E2Fa has been shown to be an essential LRI component by regulating the cell division (Berckmans et al., 2011). In addition, the Inhibitor-Interactor of CDK/Kip-Related Protein2 (ICK/KRP2) participates in LRI by blocking the G1-to-S transition in XPP cells (Himanen et al., 2002; Sanz et al., 2011).

During LRI, the founder cells divide first anticlinally and then periclinally. The first anticlinal division of the founder cells is asymmetric, producing a short and a long daughter cell at the center and flanking the new LRP, respectively. This event is followed by periclinal asymmetric divisions, giving rise to a second layer within the LRP (Malamy and Benfey, 1997; De Smet et al., 2008). After the first asymmetric cell divisions, an auxin response maximum can be observed in the central core of small cells (Benková et al., 2003) (Fig 2).

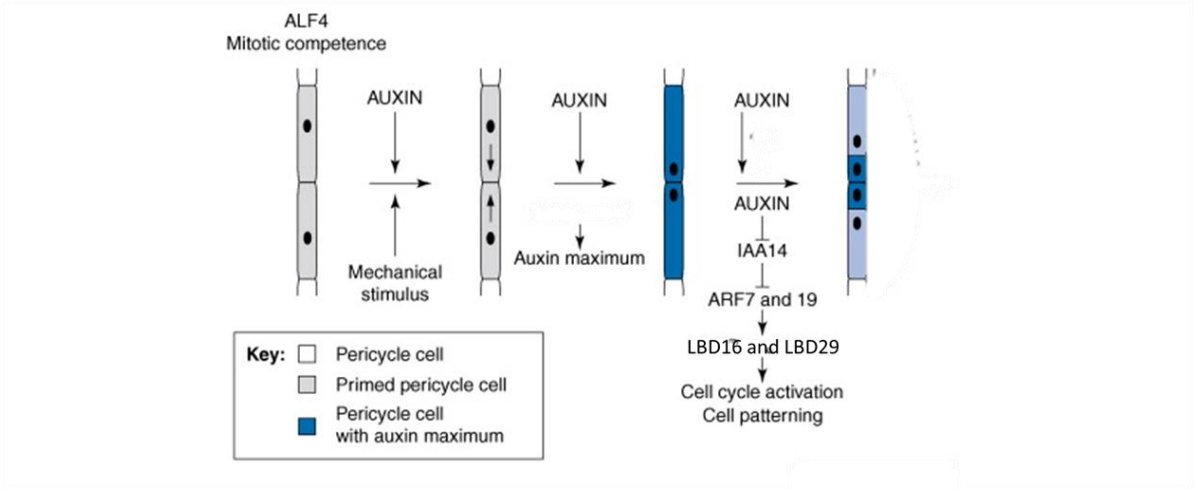

Figure 2. Early events in the LRI process. Abbreviations: ALF4, ABERRANT LATERAL ROOT FORMATION4; ARF, AUXIN RESPONSE FACTOR; LBD, LATERAL ORGAN BOUNDARIES-DOMAIN. This figure was modified from Péret et al., 2009.

Downstream of the auxin maximum, the SLR1/IAA14 repressor plays a central role. A gain-of-function *slr-1* mutant is defective in LRP formation because the founder cells fail to divide (Fukaki et al., 2002). Overexpression of the D-type cyclin (CYCD3;1) in a *slr-1* background could rescue complete rounds of cell division in the pericycle, but did not lead to the formation of LRs (Vanneste et al., 2005), suggesting that cell division activation is not sufficient to rescue LR formation in *slr-1*. These data indicate that SLR1/IAA14 is necessary for cell division, but also for founder cell identity specification. In contrast, the loss-of-function *iaa14-1* mutant had no obvious LR phenotype (Okushima et al., 2005), indicating that other members of the AUX/IAA family might act redundantly to control LRI. The *arf7arf19* double mutant phenocopies the *slr-1* mutant (Ditengou et al., 2008), suggesting that SLR1/IAA14 acts on ARF7/ARF19 to control LRI. Indeed, SLR1/IAA14 has been demonstrated to interact directly with ARF7 and ARF19 (Fukaki et al., 2005). Target gene analysis has revealed that LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18 (LBD16/ASL18) and LBD29/ASL16 are controlled by ARF7 and ARF19 (Okushima et al., 2007; Goh et al.,

2012) and, recently, that LBD18/ASL20 also participates in the regulation of LRI via interaction with LBD16/ASL18 downstream of ARF7 and ARF19 (Lee et al., 2009). Although the *arf7arf19* mutant is severely impaired in LRI, it still forms a few LRs (Okushima et al., 2005; Fukaki et al., 2005), implying that other ARF(s) might also be involved in LRI control. Indeed, also the BODENLOS (BDL)/IAA12-MONOPTEROS (MP)/ARF5 controls LRI, but at a later step than SLR/IAA14-ARF7/ARF19 module (De Smet, 2010).

1.3 Lateral root primodium formation and development

Following LRI, rapid anticlinal, periclinal, and tangential divisions generate a dome via strictly coordinated cell division and differentiation patterns. With histology and histochemistry methods, the process of LRP development was analyzed in depth and can be divided into eight stages (stage I to VII and emergence) (Malamy and Benfey, 1997). Briefly, stage I consists of single layered primordia composed of up to 10 small cells of equal length. In stage II, periclinal division results in two layers of cells, forming an inner and outer layer. Next, the outer layer divides periclinally, generating a three-layer primordium (stage III). Stage IV consists of four cell layers due to periclinal divisions in the inner layer. At this stage, the LRP has penetrated the parent endodermal layer. Stage V is characterized by anticlinal division of central cells in the outer layers 1 and 2. At this stage, LRP is midway through the parent cortex. Further anticlinal and periclinal divisions create stage VI. The LRP of this stage has passed through the parent cortex layer, has penetrated into the epidermis, and begins to resemble the mature root tip. After stage VI, at stage VII, enlargement of the primordium makes it difficult to still distinguish particular divisions. Many of the LRP cells continue to divide anticlinally, resulting in merging from the parent root (Fig 3).

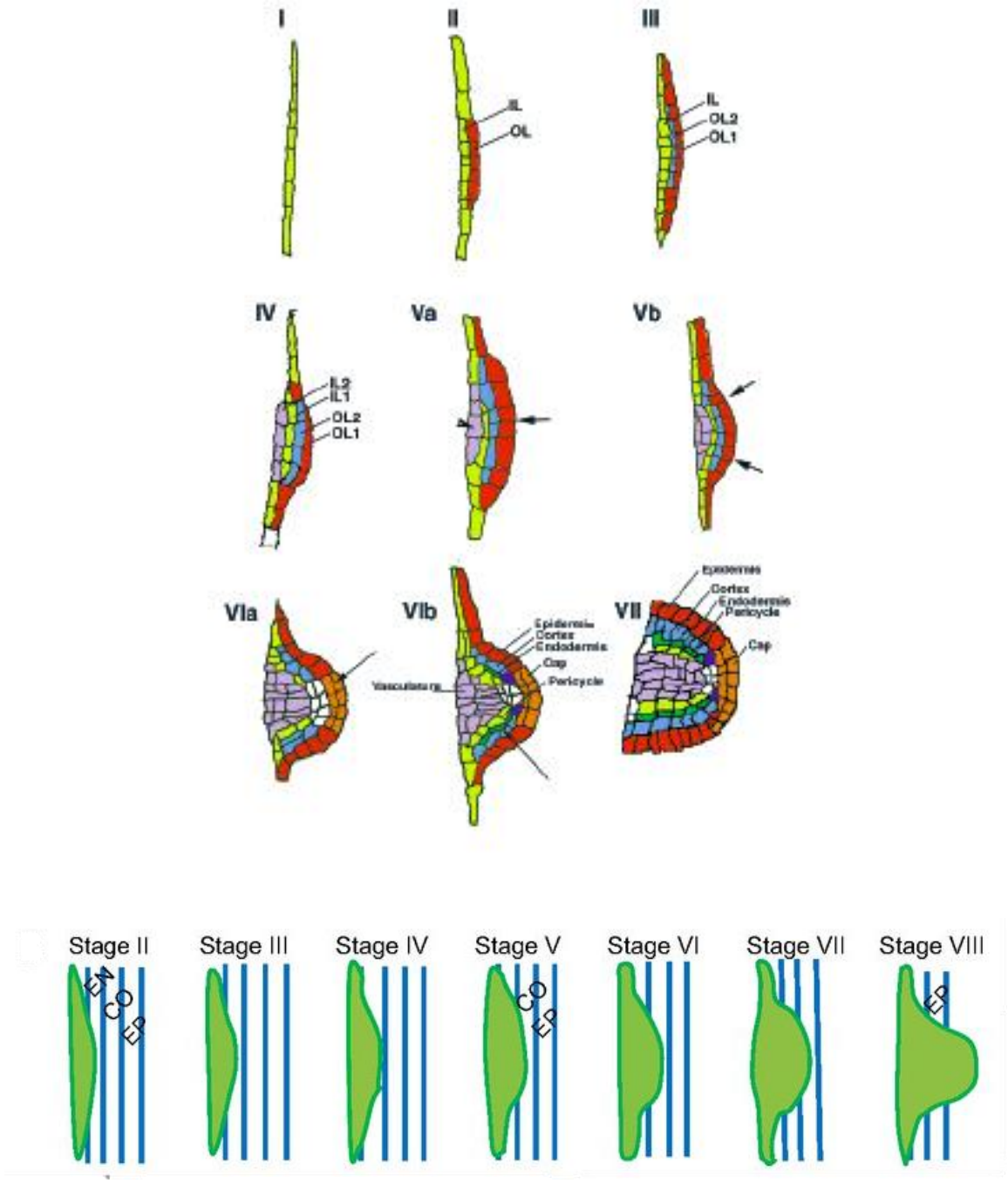

Figure 3. Stages in the lateral root formation. abbreviation: IL, inner layer; OL, out layer; EN, endodermis; CO, cortex; EP, epidermis. These figures were taken respectively from Malamy & Benfey (1997) and Kumpf et al, (2013).

Figure 3. Stages of LR formation. Abbreviation: IL, inner layer; OL, out layer; EN, endodermis; CO, cortex; EP, epidermis. These figures were taken from Malamy and Benfey (1997) and Kumpf et al. (2013).

During lateral root development, auxin is a key patterning regulator and a graded auxin distribution together with auxin maxima at the primordia tips coordinate cell division and differentiation (Benková et al., 2003). PIN-FORMED (PIN) proteins that are auxin efflux carriers play a main role in the establishment of auxin gradients and are also required for lateral root development. Mutations in multiple members of the *PIN* family disrupt auxin-induced LRP development due to interference with the establishment of auxin gradients (Benková et al., 2003). During lateral root development, first auxin accumulates at the LRI site that is mediated by PIN-dependent auxin transport from the root tip and that results in cell division activation. Subsequently, endogenous signals will mediate retargeting of continuously cycling PIN proteins to redirect auxin transport to provide auxin from the root vasculature through the interior of the primordium into the LR tip, while auxin is transported back via the outer layer from the LR tip (Benková et al., 2003).

In addition to auxin, two other factors have been identified that are also involved in the control of lateral root organogenesis. *ARABIDOPSIS CRINKLY4* (*ACR4*) is a marker gene of the short daughter cells that are generated after the first asymmetric division of LR founder cells (De Smet et al., 2008). The double *acr4 crinkly4-related3* (*ccr3*) and *acr4 ccr4* mutants show an increased number of LRP, fail to express the LBD5 marker in the LRP flanks, and sometimes exhibit fused LRP. Because *ACR4* encodes a membrane-associated receptor-like kinase, it is supposed to regulate LRP patterning by recognizing a novel class of signaling molecules. PUCHI, encoding an APETALA2 (AP2)-like transcription factor, is induced by auxin at the LRP flanks (Hirota et

al., 2007). In the *puchi-1* loss-of-function mutant, cells in the flanks undergo additional divisions, demonstrating a role for this factor in regulating cell behavior at the LRP flanks (Hirota et al., 2007). Hence, both ACR4 and PUCHI control the LRP boundaries.

To become a real LR, LRP have to pass through several intervening primary root tissues. In *Arabidopsis*, due to its simple root anatomy, LRP have to break through only three overlying outer layers of cells (endodermis, cortex, and epidermis). During LRP emergence, endodermal cells adjacent to the LRP can undergo tangential or anticlinal divisions, but the new endodermal cells do not form a Casparian strip that is beneficial for continued LRP division (Péret et al., 2009; Vermeer et al., 2014). Auxin released by the LRP induces a SHY2/IAA3-based signaling cascade in the endodermis and an SLR/IAA14-ARF7-ARF19 signaling cascade in the cortex and epidermis that would facilitate emergence (Swarup et al., 2008; reviewed by Péret et al., 2009; Vermeer et al., 2014). Functional analysis of auxin importers of *AUX1/LIKE AUX* (*LAX*) family members have shown that *LAX3* that is expressed in the outer endodermis and cortex cells, adjacent to the growing LR promotes LR emergence by affecting the auxin influx into these outer cell layers (Swarup et al., 2008). Thus, LRP-produced auxin induces the expression of the neighboring cells, resulting in a positive feedback loop that will lead to high auxin levels proposed to result in the accumulation of cell wall-remodeling enzymes to promote cell separation. This conclusion is based on the fact that several genes encoding cell wall-remodeling enzymes are regulated in a LAX3-dependent manner in the outer cell layers of the parent root (Laskowski et al., 2006; Swarup et al., 2008; Péret et al., 2009).

2. Plant Hormones in Lateral Root Development

2.1 Auxin

Auxin was first discovered because of its involvement in response to light (Went, 1926). The name auxin is derived from the Greek “auxein”, meaning to increase or to grow and nowadays auxin has been accepted as a central key player in the regulation of plant growth and development as well as in response to abiotic environment changes. As already clear from above, it also plays an essential role in controlling various aspects of root growth (reviewed by Overvoorde et al., 2010; Saini et al., 2013).

2.1.1 Auxin Biosynthesis

In contrast to the great progress in auxin signaling and transport (reviewed by Quint and Gray 2006; Petrášek and Friml, 2009; Cardarelli and Cecchetti, 2014), less is known about auxin production in plants. IAA, IBA, 4-chloroindole-3-acetic acid, and phenylacetic acid are the main natural auxins, of which IAA is broadly used in science (Ludwig-Müller, 2000; Katayama, 2000; Zolman et al., 2008; Simon et al., 2011; Tivendale et al., 2012).

Auxin can be produced from multiple biosynthesis pathways. In addition to release from IAA conjugates (Korasick et al., 2013), two major biosynthesis routes have been confirmed by biochemical and genetic studies. One group contains the tryptophan (Trp)-dependent and the other the Trp-independent pathways (Strader and Bartel 2008; Zhao, 2010; Mano and Nemoto, 2012; Ljung, 2013). Four Trp-dependent IAA biosynthesis pathways have been characterized: (i) the indole-3-acetamide (IAM) pathway; (ii) the indole-3-pyruvic acid (IPA) pathway; (iii) the tryptamine (TAM) pathway; and (iv) the indole-3-acetaldoxime (IAOX) pathway (Mashiguchi et al., 2011; Mano and Nemoto, 2012) (Fig. 4). In the other pathway, YUCCA (YUC) was identified because the phenotypes of the mutant *yuc1-D* that shows a long hypocotyl, a short

primary root, increased root hair numbers and apical dominance were very similar to those of the known auxin overproduction mutants (Zhao et al., 2001). Furthermore, *yuc1-D* is resistant to toxic Trp analogs, indicating that YUCCA participates in auxin production by a Trp-dependent pathway.

In contrast to the Trp-dependent pathways, IAA biosynthesis from Trp-independent pathways is still to be fully discovered (reviewed by Ljung, 2013). The *Arabidopsis* mutants *trp3-1* and *trp2-1*, which are defective in Trp synthase, can still accumulate IAA conjugates, suggesting that IAA might be produced from Trp precursors without the production of Trp (Normanly et al., 1993). In addition to biosynthesis, plants can also get IAA from other sources, such as IAA conjugates and IBA (reviewed by Strader and Bartel, 2011; Ljung, 2013). IAA conjugation is used by plant cells to quickly reduce or enhance the auxin concentrations in a reversible way.

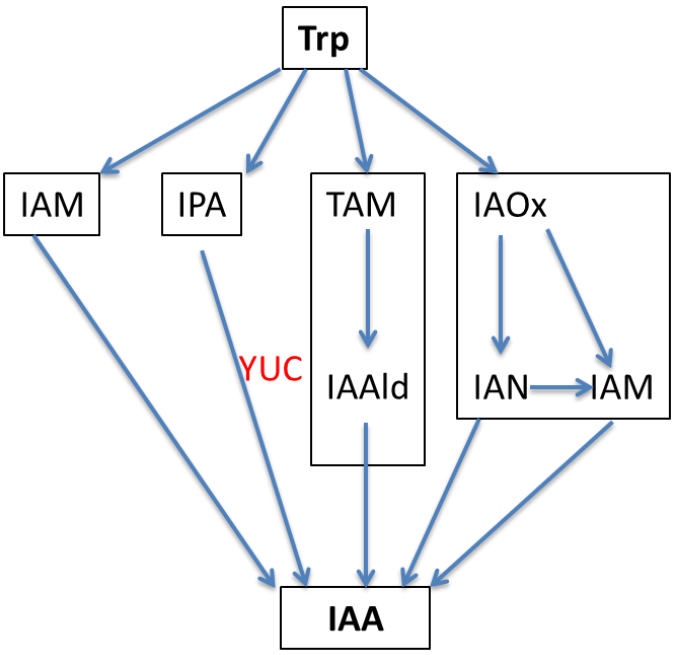

Figure 4. Trp-dependent pathways of auxin biosynthesis. In the indole-3-acetamide (IAM) pathway, IAM converts into IAA via IAM hydrolases. In the indole-3-pyruvic acid (IPA) pathway, Trp is converted into IPA, then decarboxyled into IAA by YUCCA (YUC). In the TAM pathway, Trp is converted into

tryptamine (TAM), which is then transformed into indole-3-acetaldehyde (IAAld) in pea (*Pisum sativum*), resulting in IAA production. In the indole-3-acetaldoxime (IAOx) pathway, IAOx can be transformed into indole-3-acetonitrile (IAN) and IAM to produce IAA. The figure adapted from Mashiguchi et al., 2011.

2.1.2 Auxin Signaling

Auxin signaling involves two separate interconnected pathways, one includes transcriptional responses, whereas the other acts independently of transcription and is still not well characterized. The nucleus-localized receptors, F-box proteins TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN SIGNALING F-BOX (AFB), activate the transcriptional response (Dharmasiri et al., 2005a, b; Kepinski and Leyser, 2005; Tan et al., 2007). The TIR1/AFB proteins are part of the SCF E3 ubiquitin ligases that can bind auxin whereafter target proteins are polyubiquitinated, a signal that serves for 26S proteasomal degradation (Dos Santos Maraschin et al., 2009). The target proteins to be degraded are the Aux/IAA proteins that act as negative regulators. The *AUX/IAA* gene family consists of 29 genes in *Arabidopsis*, among which most members are upregulated by auxin (Remington et al., 2004). Downstream of the AUX/IAA proteins, the Auxin Response Factor (ARF) transcription factors control auxin-mediated transcriptional changes by binding the Auxin Response Elements (AREs) within the promoter of auxin regulated genes (reviewed by Quint and Gray, 2006). As shown in Fig 5, in the absence of auxin or at low auxin levels, Aux/IAA and ARFs heterodimerize, resulting in a repression of the ARF transcriptional activity (reviewed by Quint and Gray, 2006). In addition, under these conditions, Aux/IAA proteins interact with the co-repressors TOPLESS (TPL) to repress auxin-dependent gene expression (Szemenyei et al., 2008; Causier et al., 2012; Hao et al., 2014). In the presence of high auxin concentrations, auxin acts as a molecular glue to promote the interaction between $SCF^{TIR1/AFB}$ and Aux/IAA repressors, thereby stimulating their

ubiquitination to target them for 26S proteasomal degradation. As a result, Aux/IAA repression of ARFs is released, activating their transcriptional activity on the downstream response genes (Kepinski and Leyser, 2005; Dharmasiri et al., 2005a). Due to the fact that the TIR1/AFB family and the Aux/IAA family comprise multiple members and due to the observed diversity and specificity of the auxin response, different combinations of $SCF^{TIR1/AFB}$ and Aux/IAA members have been suggested to form co-receptor complexes with a wide range of auxin-binding affinities that might be largely determined by the Aux/IAA repressors (Calderon-Villalobos et al., 2012; Pierre-Jerome et al., 2013).

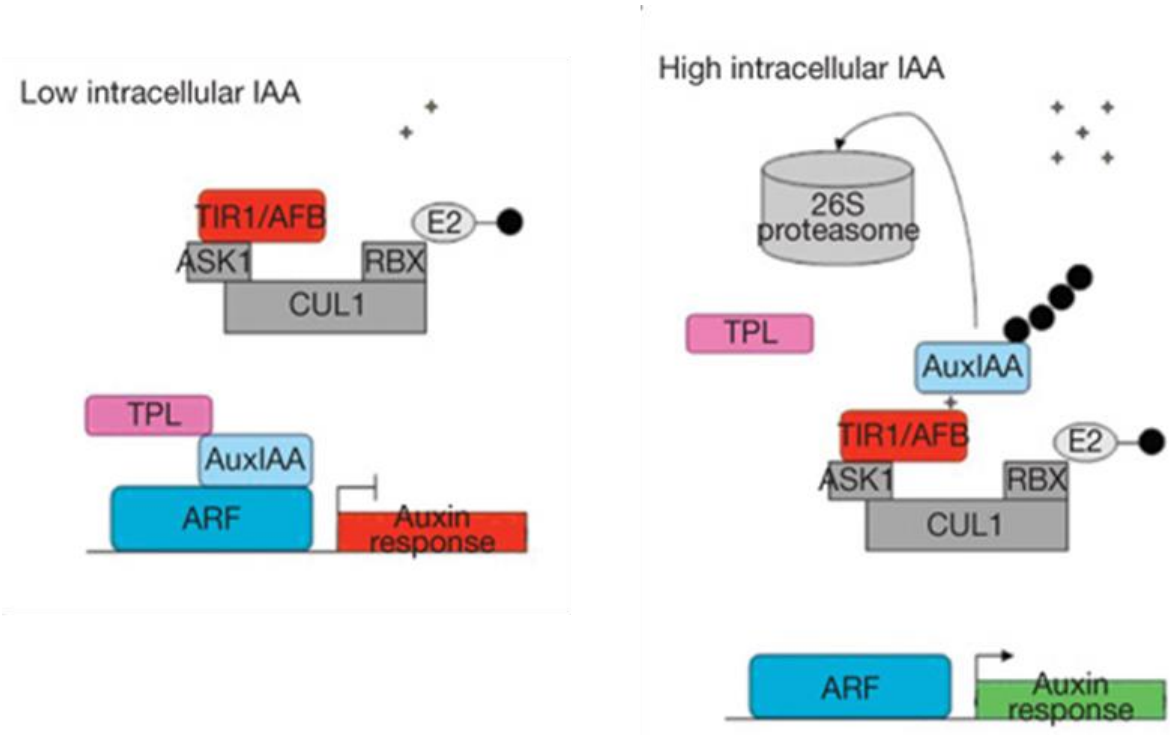

Figure 5. Model of auxin signaling pathway. In the absence or at low concentration of IAA, Aux/IAA together with TOPLESS (TPL) repress Auxin Response Factors (ARFs), leading to the inhibition of the auxin response gene expression. In the presence of auxin, The SCF complex containing TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN SIGNALING F-BOX (AFB) binds Aux/IAA repressors to ubiquinate them for proteasomal degradation, releasing ARFs and activating downstream gene expression. The figure was adapted from Santner and Estelle, 2009.

In *Arabidopsis*, there are 23 ARFs, but only five (ARF5, ARF6, ARF7, ARF8, and ARF19) act as activators that interact with the majority of Aux/IAA proteins (Ulmasov et al., 1999). The remaining 18 ARFs act as transcriptional repressors and do not or interact with Aux/IAA proteins in a limited manner (Vernoux et al., 2011).

In addition to the TIR1/AFB receptors, the S-PHASE KINASE-ASSOCIATED PROTEIN2 A (SKP2A) has also been demonstrated to bind auxin (Jurado et al., 2010; Sauer and Kleine-Vehn, 2011). The SKP2A is also an F-box protein and, upon auxin binding, has been shown to mediate proteolysis of cell cycle-related transcription factors and likewise to provide a direct link between auxin and cell proliferation and cell cycle control (Del Pozo et al., 2006; Jurado et al., 2008).

During LR development, as shown in Fig 2, auxin is required to form an auxin maximum in the primed XPP cells and the auxin-IAA14-ARF7/ARF19 and auxin-IAA12-ARF5 modules are necessary to induce LRI.

2.1.3 Auxin transport

As auxin is generally synthesized in young leaves, the main auxin transport is from the shoot to the root. However, localized and well-controlled changes in the auxin flow are required to direct plant development and control its interaction with the environment. There are two mechanisms of auxin transport, one involving long-distance transport and supporting cell-to-cell transport, also known as polar auxin transport (PAT). One of the main features of auxin transport is its directionality (polarity). For the long-distance transport, phloem is considered the place for a

natural, unregulated flow to transport most of the IAA. The cell-to-cell transport is slower than the the long-distance transport and is regulated by several carriers (Marchant et al., 2002). The influx carriers are responsible for uptake of auxin into cells, whereas efflux carriers transport auxin outside the cells. The model for auxin short-distance transport is shown in Fig 6. Owing to the auxin characteristics, an apoplastic pH between 5.5 and 5.7 favors to keep IAA in the protonated form (IAAH) that can freely enter cells. Inside the cells, due to a cytosolic pH of approximately 7.4, the protonated form quickly changes into the nondiffusible IAA anion (IAA-) that cannot easily pass through the cellular membrane. Hence, the pH difference will determine the directional diffusion of IAAH into the cells (Fig. 6). Although auxin is able to diffuse, active import inside the cells is also important for the auxin flow. Indeed, in the *Arabidopsis* mutant *auxin resistant 1* (*aux1),* poor import of the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D) into cells has been reported, whereas import of another synthetic auxin, naphthalene acetic acid (NAA), was unaffected. These data led to the recognition of AUX1 as an important auxin importer for selective auxins (Yamamoto et al., 1998). Three other influx carriers have been identified, LAX1, LAX2, and LAX3 that are similar to AUX1 and are considered as the major auxin influx carriers together with AUX1 (Swarup et al., 2008; Peret et al., 2012). Genetic and biochemical studies suggested that each member of the AUX/LAX family has specific functions in the different organs and tissues. For instance, AUX1 regulates root gravitropism, root hair development, leaf phyllotaxy, phloem “loading”, and LR emergence as well as apical hook formation together with LAX3, whereas LAX2 regulates vascular development in cotyledons. AUX1 together with LAX1, and possibly with LAX2, are involved in leaf phyllotactic patterning (Bennett et al., 1996; Stone et al., 2008; Swarup et al., 2008; Bainbridge et al., 2008; Jones et al., 2009; Péret et al., 2012, reviewed by Swarup and Péret., 2012). The function of LAX3 in LR emergence has been discussed above (Swarup et al., 2008).

After auxin has entered the cells, it cannot easily diffuse out of the cells. Its export needs the assistance of transmembrane efflux carriers and the asymmetric localization of these efflux carriers has been proposed to determine the polarity of auxin movement (Petrášek et al., 2006; Wiśniewská et al., 2006). Different classes of auxin export carriers were identified: the PIN proteins, the PIN-LIKES (PILK) proteins, and members of the ATP-binding cassette B (ABCB), also known as multidrug resistance or P-glycoprotein (MDR/PGP) subfamiliy of ABC transporters (Cho et al., 2007; Růžička et al., 2010; Barbez et al., 2012). The auxin transport inhibitor NPA could directly or indirectly inhibit these proteins (Zettl et al., 1992; Muday et al., 1993; Sundberg et al., 1994). PIN proteins are key components to control PAT. These proteins are produced in a tissue-specific and cell-specific manner and are asymmetrically localized at the plasma membrane, in a pattern that seems to be determined by auxin itself (Friml et al., 2003; Paponov et al., 2005; Friml, 2010). In *Arabidopsis*; there are eight PIN proteins that, based on a hydrophilic loop, can be divided into two groups: large-loop and short-loop subgroups. Large-loop PIN proteins include PIN1, PIN2, PIN3, PIN4, and PIN7 that are localized in the plasma membrane and direct auxin transport through the plasma membranes. In contrast, PIN5, PIN6, and PIN8 are short-loop PIN proteins, are not recruited to the plasma membrane, but mediate auxin homeostasis between cytoplasm and endoplasmic reticulum (ER) (Mravec et al., 2009; Wabnik et al., 2011). The PILK proteins are localized in the ER and are also described as regulators of intracellular auxin homeostasis (Barbez et al., 2012).

The tissue- and cell-specific expression patterns of the PIN proteins, their asymmetric localization on membranes as well as the strong regulation of their abundance at the plasma membranes (Mravel et al., 2009; Bosco et al., 2012) provide well-controlled auxin flows that underpin plant development as well as plasticity to quickly adapt to changing biotic and abiotic environments (Friml et al., 2002a, 2002b; Benková et al., 2003; Tanaka et al., 2006). As an

example, root growth is controlled by a distal auxin maximum that correlates with pattern formation and orientation and extent of cell division, but these processes are strongly affected by PAT inhibition (Sabatini et al., 1999). At the root meristem, PIN1, PIN3, PIN4, and PIN7 are localized in the stele cells, resulting in auxin flow from the upper root part to the quiescent center. PIN2 localized on the apical side of epidermal cells directs the "bottom" auxin upward to the end of meristem zone where PIN1, PIN3, and PIN7 can recycle auxin to the stele, thus forming an auxin flow loop (Fig. 6), in which PIN2 is a main component for mediating proximal (basipetal) auxin transport (Blilou et al., 2004). Interrupting this flow loop, such as by *PIN2* mutation or *pin2*-containing multimutations, resulted in the reduction in root length and root meristem size (Blilou et al., 2004).

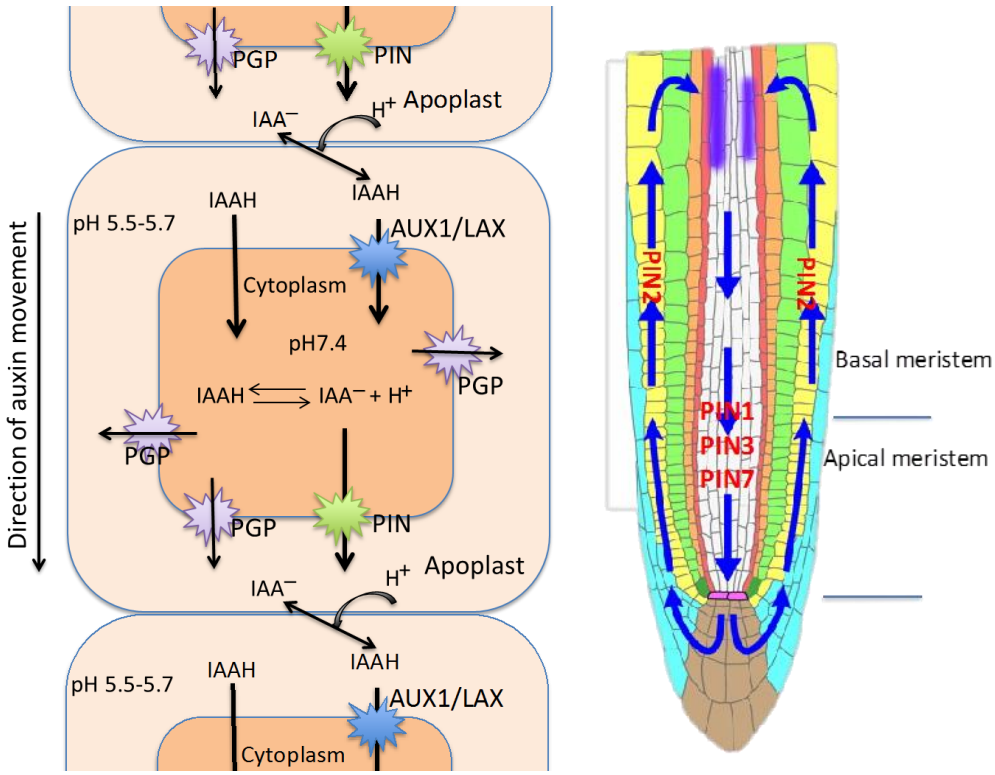

Figure 6. Module of polar auxin transport (PAT). Left, PAT occurs in a cell-to-cell manner and depends on specific influx (AUX1/LAX) and efflux (PIN andPGP) carriers. **Right**, auxin flow loop in primary root

meristem, which is critical for primary root growth and LRI. Figures adapted from Robert and Friml, 2009 (left) and Overvoorde et al., 2010 (right).

Similarly, the auxin flow loop shown on the right of Figure 4 is also important for LRI. As discussed above, priming XPP cells happens in the basal meristem and these founder cells become primed due to an auxin response maximum arising in the neighboring protoxylem cells (De Smet et al, 2007). Moreover, the IAA transport inhibitor NPA has revealed that IAA movement from the root tip is essential for LRI (Casimiro et al., 2001). In LR formation, shoot-derived auxin pool is also involved. In the absence of shoot-derived IAA in *shoot meristmeless1* (*stm1*) mutants, an equal wild-type number of LRP could be observed, while the LR emergence was blocked (Casimiro et al., 2001). Other studies have also shown that phloem-based IAA transport from the leaf to the root at the seedling stage is essential for the LR emergence and that genetic or pharmacological manipulation of this auxin flow disrupts LR formation (Reed et al., 1998; Bhalerao et al., 2002; Wu et al., 2007).

2.2 Cytokinin

The discovery of cytokinins (CKs) dates back to 1955, when a substance, designated kinetin, was isolated from herring sperm that could stimulate cell division in plants (Miller et al., 1955). Several years later, *trans*-zeatin (tZ) was identified and subsequently followed by many other CK types (Miller, 1961; reviewed by Mok and Mok, 2001). CKs are a class of plant growth substances that promote cell division in plant roots and shoots and that are mainly synthesized in the meristematic root regions, although young leaves and embryos can also serve as CK sources. They are involved in various aspects of plant development, ranging from organ formation and

apical dominance to leaf senescence. Naturally occurring CKs can be divided into two groups based on their side chain: adenine-type CKs with isoprene-derived side chains represented by kinetin, N6-(Δ 2-isopentenyl)-adenine (iP), tZ, *cis*-zeatin (cZ), dihydrozeatin (dZ), and 6-benzylaminopurine (BAP); and phenylurea-type CKs with aromatic side chains, such as diphenylurea and thidiazuron (Sakakibara, 2006; Mok and Mok, 2001). CKs have also been reported to be important for various aspects of root development (Werner et al., 2003; Dello Ioio et al., 2007, 2008; Muller and Sheen, 2008; Kuderova et al., 2008)

2.2.1 Cytokinin biosynthesis

CK biosynthesis is a multistep process requiring the activity of several enzymes. Adenosine phosphate-isopentenyl transferase (IPT) catalyzes the first reaction in this pathway by means of ATP, ADP, or AMP as substrates and dimethylallyl diphosphate (DMAPP) as prenyl donors, producing iP nucleotides (Keiber and Schaller, 2014; Sakamoto et al., 2006) (Fig. 7). These iP nucleotides can undergo further hydroxylation to produce tZ nucleotides that are catalyzed by two cytochrome P450 monooxygenase (CYP735A1 and CYP735A2) in *Arabidopsis* (Takei et al., 2004; Kiba et al., 2013). Finally, enzymes from the LONELY GUY (LOG) family convert the inactive CK precursors into active free-base forms (Kurakawa et al., 2007). The *Arabidopsis* genome encodes seven *IPT* genes (*AtIPT1*, *AtIPT3*, *AtIPT4*, *AtIPT5*, *AtIPT6*, *AtIPT7*, and *AtIPT8*) (Kakimoto, 2001; Takei et al., 2001) and each one has a unique spatial expression pattern (Miyawaki et al., 2004). For example, *AtIPT1* is expressed in the root tip, leaf axils, and immature seeds; *AtIPT5* is expressed in root primordia, columella root caps, and other vegetative organs; *AtIPT7* is expressed in the root elongation zone, young leaves, and pollen tubes. The *Arabidopsis* genome encodes seven *LOG* genes and these genes also show tissue- and organ-specific expression (Kuroha et al., 2009). For instance, *LOG7* and *LOG4* play a major role in the

shoot apical meristem, whereas *LOG3* and *LOG4* act in the root (Tokunaga et al., 2012). Another pathway to synthesize CK is via tRNA prenylation catalyzed by tRNA-IPTs (Miyawaki et al., 2006). In *Arabidopsis*, two tRNA-IPTs, AtIPT2 and AtIPT9, catalyze isopentylation of tRNA. Mutants deficient in both tRNA-IPTs resulted in an undetectable low cZ content, whereas the levels of iP and tZ CKs were not affected (Sakamoto et al., 2006), indicating that the tRNA-IPT pathway is the main route to supply cZ.

Similar to auxin, CK homeostasis is essential for plant development. CK levels are tightly controlled, not only via regulation of synthesis, but also by equilibrated degradation through CK oxidase/dehydrogenase (CKX) or conjugation to glucose (reviewed by Frebort et al., 2011). Glucosyl conjugates are inactive in bioassays and do not bind to the CK receptors (Spichal et al., 2004). In *Aradidopsis*, seven *CKX* genes were found of which the expression is induced rapidly upon CK treatment (Bhargava et al., 2013). Overexpressed *CKX* genes exhibit low CK levels, resulting in phenotypes of CK deficiency (Werner et al., 2003). Among the *CKX* genes, some of them show unique spatial expression patterns. For instance, *CKX2* and *CKX6* are expressed in the central cylinder, whereas *CKX1* is expressed not only in the central cylinder, but also in the shoot apex and pericycle cells, in which LRI occurs. In contrast, the root cap and root apex are the sites for *CKX4* and *CKX5* expression. Furthermore, *CKX5* is also detected in LRP.

This spatial distribution of IPT, LOG, and CKX together with the observation that various CK species accumulate in the xylem and phloem indicate that CKs are produced in a tissue/cell-specific manner and that the levels are extremely regulated.

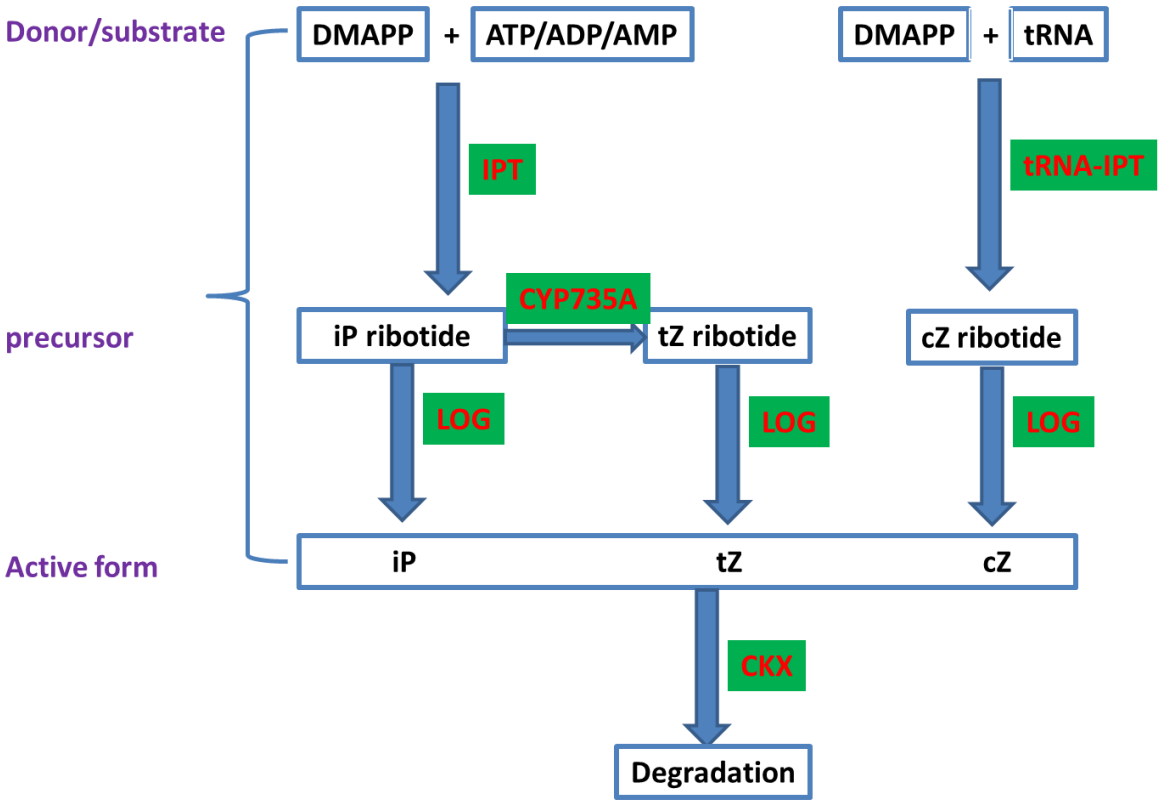

Figure 7. Model for cytokinin biosynthesis. The iP and tZ CK types are initiated by adenosine phosphate-isopentenyl transferases (IPTs), resulting in iP ribotide. With CYP735A enzymes, iP ribotide can be converted to tZ ribotide. cZ CKs are synthesized in *Arabidopsis* exclusively by tRNA-IPTs that use tRNAs as prenyl acceptors. All these CK precursors can be catalyzed into corresponding active CK forms by 5'-monophosphate phosphoribohydrolases (LOGs). Figure adapted from Kudo et al., 2010.

2.2.2 Cytokinin transport

Based on the spatial expression patterns of the genes involved in CK biosynthesis, degradation, and signaling (Takei et al., 2004; Miyawaki et al., 2006; Werner et al., 2006), CKs have been suggested to act as autocrine or paracrine signals. In contrast, CKs were found in the xylem and phloem sap, supporting the hypothesis that they can function also as long-distance signals (Kudoyarova et al., 2007; Foo et al., 2007; Kudo et al., 2010). This hypothesis was confirmed by grafting experiments between wild-type and CK biosynthesis mutants (Matsumoto-Kitano et al.,

2008). Currently, a model has been proposed that shows that long-distance CK transport occurs in the vascular tissues with differential directions depending on the CK type involved: tZ ribosides would be transported in the xylem upward toward the shoot, whereas iP-type CKs would be transported downward into the root (Matsumoto-Kitano et al., 2008; Kudo et al., 2010; Bishopp et al., 2011). However, the mechanism of long-distance CK transport remains unknown. In *Arabidopsis*, the purine permease (PUP) protein family and the equilibrative nucleoside transporter (ENT) family have been proposed as CK transport candidates (Burkle et al., 2003; Sun et al., 2005; Hirose et al., 2008). Among the CK types, the PUP family has been shown to be responsible for the active uptake of tZ and iP, whereas the ENT family was involved in the uptake of iP ribosides and tZ ribosides (Burkle et al., 2003; Li et al., 2003; Hirose et al., 2005). Recently, the *Arabidopsis* ABC transporter G14 (ABCG14) has been identified to be mainly expressed in roots and essentially for delivery of tZ-type CKs to shoots via the xylem (Ko et al., 2014; Zhang et al., 2014).

2.2.3 Cytokinin signaling

The CK signal transduction pathway (Fig. 8) involves a phosphorelay system that is similar to the bacterial two-component signaling system. Two conserved proteins, a histidine (His) kinase sensor and a response regulator (RR) protein are central within the signaling cascade (El-Showk et al., 2013). In addition to these proteins, the phosphorelay cascade that connects these two regulators involves histidine phosphotransferase (HP) proteins. In short, after CKs have bound to the His kinase receptors, the His kinases are autophosphorylated. Next, an intramolecular transfer of the phosphoryl to an Asp residue takes place and then the phosphoryl group is transferred to the histidine phosphotransfer proteins (AHPs) that mediate the cytoplasm-to-nucleus signal transfer. In the nucleus, phosphor-accepting response regulators (ARRs) receive

the phosphoryl on an Asp, modulating expression of the downstream genes (reviewed by Hwang et al., 2012).

In *Arabidopsis*, so far three His kinases, AHK2, AHK3, and AHK4, act as CK receptors (Mähönen et al., 2000; Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Hwang and Sheen, 2001). The mutants lacking all the three receptors show no CK responses and produce small infertile plants (Kinoshita-Tsujimura and Kakimoto, 2011). Studies suggested that the three receptors might have some specificity, because they show a different sensitivity to various CKs (Stolz et al., 2011; Heyl et al., 2012).

ARRs comprise a large protein family that can be classified into two types according to their functions and structures. B-type ARRs (ARR1, ARR2, ARR10 to ARR14, and ARR18 to ARR21) that belong to the Myb-transcription factor family activate the transcription of CK primary response genes (Hutchison et al., 2006; Argyros et al., 2008; Argueso et al., 2010), but ARR3 to ARR9 and ARR15 to ARR17 are A-type ARRs that contribute to a negative feedback mechanism that helps to fine-tune the function of the CK signaling pathway. The B-type ARRs promote directly the expression of the A-type ARRs and, conversely, the A-type ARRs repress the B-type ARRs activities and are stabilized by phosphorylation-mediated AHPs (To et al., 2004).

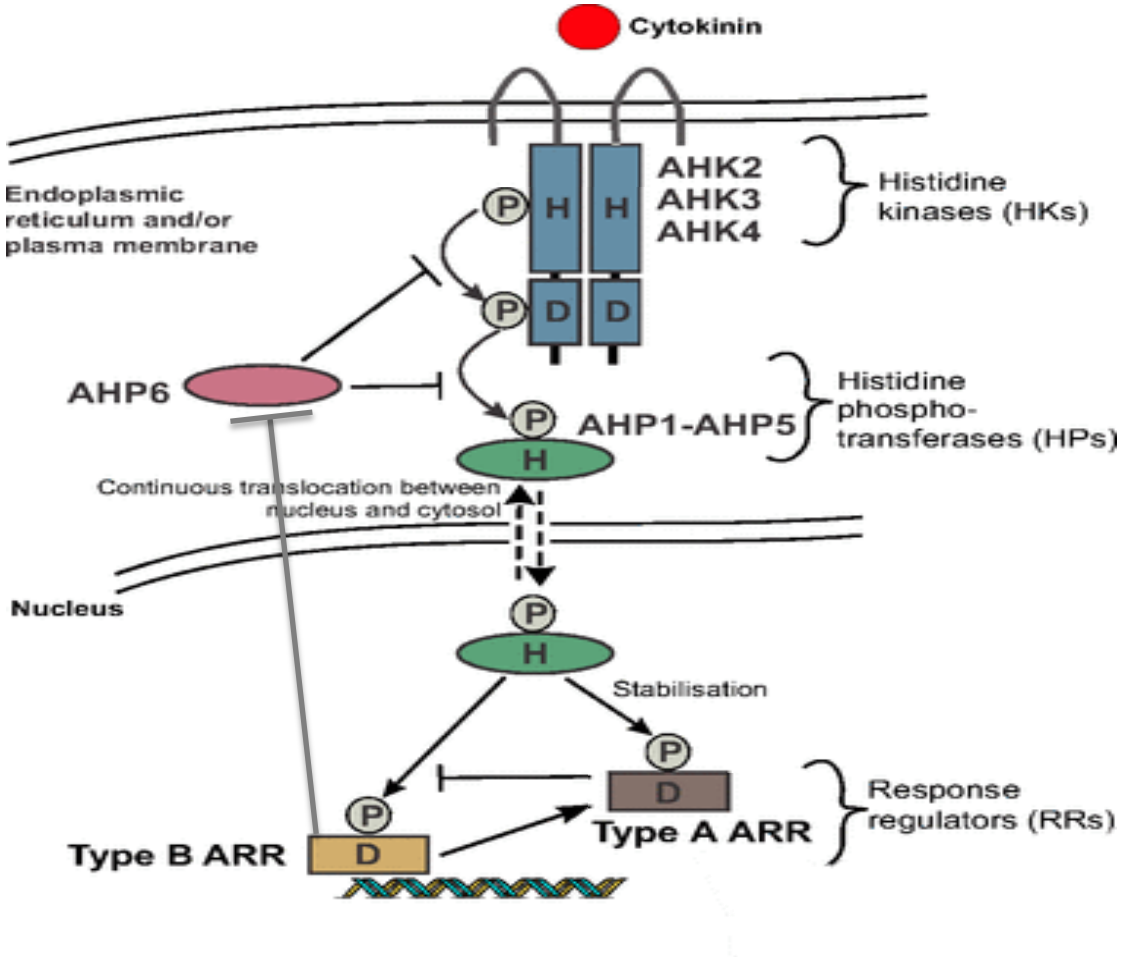

Figure 8. Model of cytokinin (CK) signaling pathway. The CK receptors *Arabidopsis* histidine kinases (AHKs) are localized on the endoplasmic reticulum as well as on the plasma membrane. CKs bind to AHK proteins, inducing a phosphoryl group (P) to transfer from a conserved His (H) to an Asp (D) residue within the receptor and is then relayed to the *Arabidopsis* histidine phosphotransferase proteins (AHP1 to AHP5). The AHPs continuously translocate the signaling to the nucleus-located *Arabidopsis* response regulators (ARRs) and are stabilized by phosphorylation of the A-type ARRs. The phosphorylated B-type ARRs can bind DNA and initiate transcription of CK-responsive genes. In addition, AHP6 inhibits CK signaling by competing with AHP1 to AHP5 for phosphotransfer. Figure is adapted from El-Showk et al., 2013.

2.2.4 The role of cytokinin in root development

In contrast to the key positive roles of auxin in LR development, CK acts antagonistically and negatively regulates LR development. The CK receptor mutants and B-type ARR mutants show more LRs than the wild type (WT) (Riefler et al., 2006; Mason et al., 2005; Hutchison et al., 2006; Chang et al., 2013) and, in agreement, transgenic plants with reduced endogenous CK content display an increased number of LRs (Werner et al., 2003). In contrast, multiple mutants in the A-type ARRs reduce the numbers of LRs (To et al., 2004). Based on these studies, endogenous CKs have been suggested to be involved in the inhibition of LR development. Further studies indicated that the negative effect of CKs on LR development happens via pericycle inhibition of founder cell cycle progression during the G2-to-M transition phase (Li et al., 2006). Furthermore, expression of *IPT* in protoxylem pericycle cells was shown to inhibit LR initiation, whereas *CKX1* expression in the same tissue cells could release the inhibition, resulting in more LRs (Laplaze et al., 2007). In addition to affecting LR development, CKs also regulate primary root growth. Application of exogenous CK or overexpression of *IPT* gene inhibit root growth and reduce meristem size of primary roots (Dello Ioio et al., 2007; Kuderova et al., 2008), but overexpression of CK level- reducing *CKX*s leads to an increase in root growth, due to enlarged meristems (Werner et al., 2003; Dello Ioio et al., 2007).

Regulation of LR development and primary root growth by CKs has been suggested to be caused by an interplay with auxin (Dello Ioio et al., 2008; Moubayidin et al., 2010). Crosstalk experiments have been executed to examine how CKs interact with auxins to influence LR development and root growth (reviewed by Aloni et al., 2006; Su et al., 2011; El-Showk et al., 2013). For instance, in the *Arabidopsis* root meristem, the Aux/IAA gene *SHY2/IAA3* was shown to be the central key in controlling meristem growth, because *ARR1* and *ARR12* activate *SHY2*, which negatively regulates *PIN* gene expression, ultimately leading to a reduced meristem size.

Conversely, auxin mediates the degradation of the SHY2 protein, sustaining PIN activities and cell division to keep meristem growth (Dello Ioio et al., 2008; Moubayidin et al., 2010).

During LR development, exogenous CKs were shown to inhibit *PIN* expression, leading to interference with the auxin gradient establishment that is required to pattern LRP, thereby inhibiting LRI (Laplaze et al., 2007). Application of CKs also affected the spatial expression of PIN proteins inside the LRP, thus preventing the formation of the auxin gradient inside the LRP (Laplaze et al., 2007). The CK signaling genes, such as *CRE1/AHK4*, *AHK3*, and *AHP6*, are required for a correct PIN1 localization via endocytic recycling of PIN1 at early stages (stage I) during LRP development (Marhavý et al., 2011; Moreira et al., 2013). Taken together, CKs have been proposed to mainly interfere with the establishment of the auxin gradient by inhibiting PIN expression in the founder cells, while they do not perturb the LR founder cell specification during LRI (Laplaze et al., 2007). Recently, CKs have been demonstrated to play also a pivotal role in polarizing PIN1 during the later stages of LRP (Marhavý et al., 2014). From stage III on, CKs enhanced PIN1 depletion at specific polar domains, thus rearranging the cellular PIN polarities and directly regulating the auxin flow direction toward the apex of the primordia. The increased auxin at this position was sufficient to promote LR development (Marhavy et al., 2014).

2.3. Jasmonate

Jasmonate (JA), in its most active form as an isoleucine derivate, regulates cell growth and biotic and abiotic stress responses (reviewed by Wasternack and Hause, 2013). Over the past years, studies on JA biosynthesis and signaling pathways in monocotyledonous and dicotyledonous plants have revealed many interesting insights into the action of this hormone..

2.3.1 Jasmonate biosynthesis

JAs are synthesized from α-linolenic acid (α-LA, mainly C18:3) and hexadecatrienoic acid (mainly C16:3) derived from the chloroplast membranes. In plants, C18:3 is the major precursor of JAs. Firstly, C18:3 is oxidized by a chloroplastic 13-lipoxygenase (13-LOX) to a 13-hydroperoxy derivative of linolenic acid (13-HPOT) that is then dehydrated by an allene oxide synthase (AOS) into epoxy-octadecatrienoic acid (also called allene oxide). Six genes encode LOX in the *Arabidopsis* genome, among which LOX2, LOX3, LOX4, and LOX6 are associated with the JA production (Bannenberg et al., 2009; Chauvin et al., 2013). The subsequent enzyme AOS, belonging to the CYP74 family of cytochrome P450 (Schaller and Stintzi, 2009), is a key enzyme in this pathway and its mutation resulted in the inhibition of JA production (Park et al., 2002).

In a next step, allene oxide cyclase (AOC) converts the unstable allene oxide into oxophytodienoic acid (OPDA) that is subsequently transferred from chloroplasts into peroxisomes where the next steps of the JA biosynthesis take place. Activity of the OPDA reductase (OPR) followed by several rounds of β-oxidation finally results in the production of (+)-7-*iso*-JA that is then released into the cytoplasm and can be further metabolized into various conjugated forms (Gfeller et al., 2010). Although several *OPR* genes have been revealed in rice, tomato (*Solanum lycopersicum*), and *Arabidopsis*, only *OPR3* is localized in the peroxisome in *Arabidopsis* and tomato, and mutations in this gene result in the inability of plants to produce JAs (Stintzi and Browse, 2000). The enzymatic activity of jasmonoyl isoleucine conjugate synthase 1 (JAR1) converts (+)-7-*iso*-JA into the (+)-7-*iso*-JA-isoleucine (JA-Ile), the bioactive form of JA (Guranowski et al., 2007). There are many other types of JA products in plants

(shown in Fig. 9), but it is not yet known whether all these types of JAs are biologically active or rather function as storage form of JA-IIe (Gfeller et al., 2010).

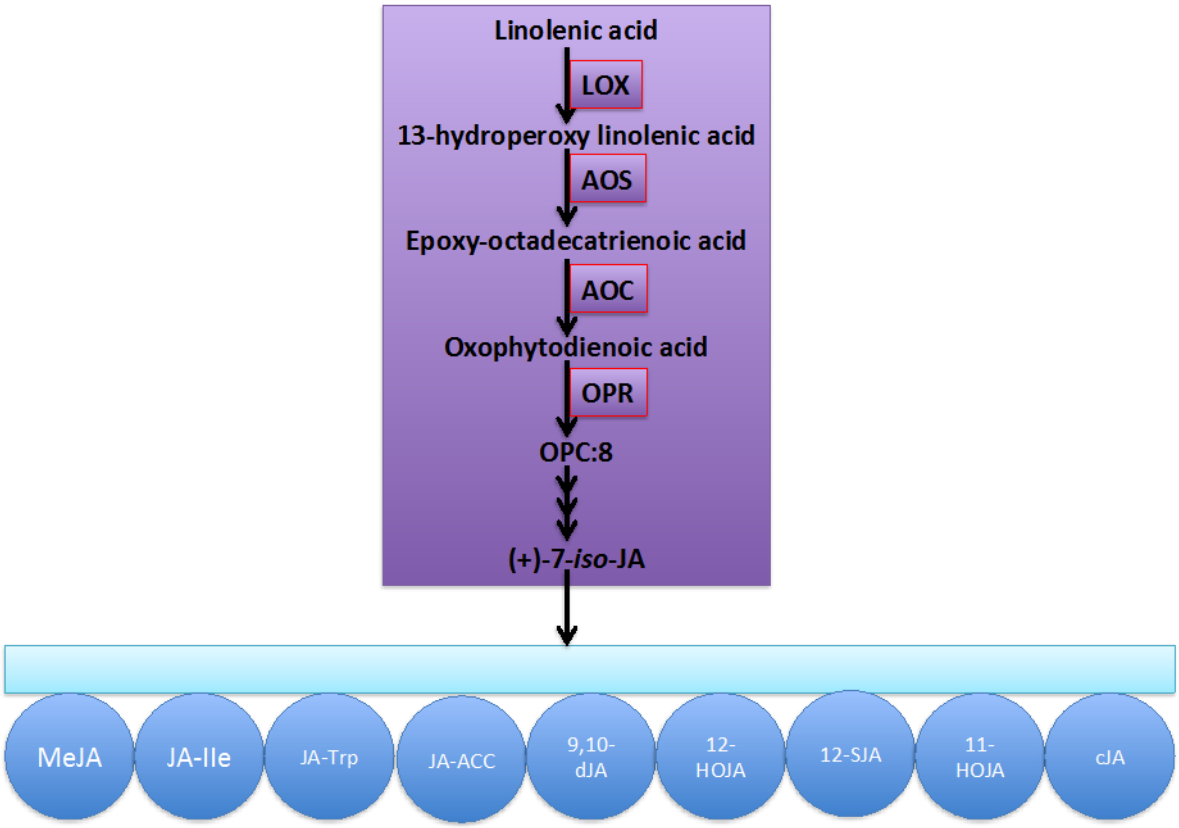

Figure 9. Model of jasmonate biosynthesis. The enzymes and the intermediates are indicated as LOX for lipoxygenase, AOS for allene oxide synthase, AOC for allene oxide cyclase, OPR for 12-oxophytodienoate reductase, and OPC8 for 3-oxo-2-(2'-[Z]-pentenyl)-cyclopentane-1-octanoicacid. The blue circles indicate some different types of jasmonate: MeJA, methyl jasmonates; JA-Iie, jasmonoyl-isoleucine; JA-Trp, tryptophan conjugate of jasmonic acid; JA-ACC , jasmonoyl-1-amino-1-cyclopropane carboxylic acid; 9,10-dJA, 9,10-dihydro jasmonic acid; 12-SJA, 12-hydroxyjasmonate; 12-HOJA, 12-hydroxyjasmonate; and cJA, *cis*-jasmone. The figure adapted from Gfeller et al., 2010; Acosta and Farmer, 2010.

2.3.2 Jasmonate signaling pathway

The JA signaling pathway has been resolved via the screening for mutants in which the JA induced inhibition of root elongation was affected (Feys et al., 1994; Lorenzo et al., 2004; Gasperini et al 2015). For these screens, coronatine has been used, a phytotoxin produced by the bacterium *Pseudomonas syringae*, which displays a similarity in chemical structure and activity with JA-Ile (Weiler et al., 1994; Axel et al., 2004). The *Arabidopsis coronatine insensitive 1 (coi1)* mutant showed insensitivity to JAs in root elongation, and was also affected in other JA related phenotypes, such as in fertility, in secondary metabolic biosynthesis, in pest and pathogen resistance and in wound responses (Xie et al., 1998; Devoto et al., 2005). The *COI1* gene encodes an F-box protein which is closely related to TIR1 and is part of an SCF E3 ligase involved in protein ubiquitination. Hence, it was presumed that COI1 is a positive regulator of the JA pathway by ubiquitinating "repressor proteins" to target them for proteasomal degradation. The cloning of the *JASMONATE INSENSITIVE 3* (*JAI3*), later designated *JASMONATE ZIM-DOMAIN 3* (*JAZ3*), provided further vital information for this hypothesis. Indeed the *jai3* mutant, in which JAI3 lacks the C-terminal domain, displayed a dominant JA insensitive phenotype because the aberrant protein was not degraded anymore upon JA-Ile treatment (Chini et al., 2007; Thines et al., 2007; Melotto et al., 2008). Further studies indeed showed that the SCF^{COI1} complex targets JAZ repressors for ubiquitination and proteasomal degradation. Thus, when the bioactive form of JA is available, it actives the SCF^{COI1} to target the JAZ proteins for ubiquitination to send them for protesomal degradation (Figure 10).

Proteasomal degradation of the JAZ proteins would then release the positively acting transcription factors, such as MYC2, to control JA-dependent gene expression. Indeed, because JAZ proteins have no DNA-binding domains, they need to interact with other proteins to activate the JA response genes. Yeast two-hybrid assays revealed that JAZ1 and JAZ3 act together with JASMONATE INSENSITIVE 1 (JIN1), also known as MYC2 (Chini et al., 2007; Melotto et al.,

2008; Chini et al., 2009). The *MYC2/JIN1* gene encodes a basic helix-loop-helix-type (bHLH) transcription factor (TF) and depending on the JA functions in plants, MYC2 was shown to positively regulate JA-responsive genes (Kazan and Manners, 2013). Two homologous MYC2 proteins, MYC3 and MYC4, have been demonstated to enhance the MYC2-regulatory effect (Cheng et al., 2011; Fernandez-Calvo et al., 2011), illustrating the need for modular and common activities of several TFs to control JA signaling. In addition, many other TFs have been identified to interact with JAZ, such as MYB21 and MYB24, bHLH003, bHLH0013 and bHLH017 (Song et al., 2011; Fonseca et al., 2014).

The JA signaling pathway can be summarized as follows (Fig. 10): JA-Ile binds and activates the SCF^{COI1} complex, leading to specific ubiquitination and degradation of the JAZ repressors, releasing positively acting TFs, such as MYC2, to regulate JA-responsive genes. When the JA signal is absent or undetectable, the JAZ proteins are stable and repress MYC2. The repressor activity acts through the recruitment of the corepressor TOPLESS (TPL) that is connected to JAZ through Novel Interactor of JAZ (NINJA) proteins (Pauwels and Goossens, 2011).

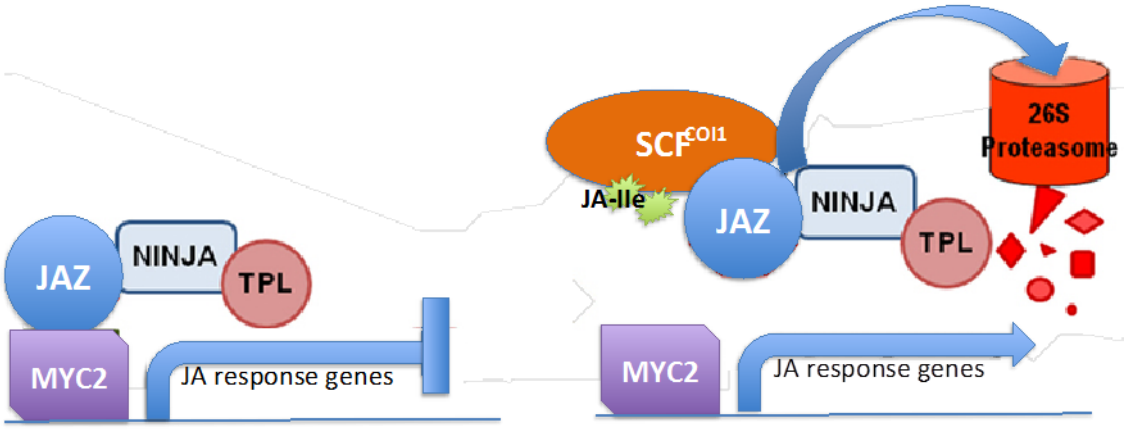

Figure 10. Jasmonate (JA) signaling pathway in the absence or presence of JA-Ile.

In the absence of JA-Ile, JASMONATE ZIM-DOMAIN (JAZ) proteins together with Novel Interactor of JAZ (NINJA) and TOPLESS (TPL) corepress the MYC transcription factors that induce JA-responsive gene

expression. When JA-Ile is available, COI1 binds to the SCF complex to ubiquitinate the JAZ to send them for degradation. As a result, the repressor activity on MYCs are released, resulting in the induction of JA-responsive genes. Figure adapted from Wager and Browse, 2012.

2.3.3 The role of jasmonate in root development

JA is known as a stress hormone, with important roles in wound and defense responses of plants. However, studies have shown that JA has also important functions in plant growth and development (Benková and Hejatko, 2009; Wasternack and Hause 2013). JA inhibits primary root growth and promotes LR and adventitious root formation (Ahkami et al 2009; Fattorini et al., 2009; Sun et al., 2009; Morquecho-Contreras et al., 2010; Raya-Gonzalez et al., 2012). Under normal growth conditions, the number of LRs could increase without impact on the primary root growth by addition of low concentrations of methyl jasmonate (MeJA), whereas the increase in LR numbers was less pronounced with inhibition of the primary root growth with high doses (Raya-Gonzalez et al., 2012). Further studies have shown that the regulation of root development by JA can be divided into mechanisms dependent and independent of auxin (Raya-Gonzalez et al., 2012). In addition, phenotypes of the JA biosynthesis or signaling mutants confirmed the role of JA in LR development (Stenzel et al., 2012; Raya-Gonzalez et al., 2012). For instance, the LR density (LRD) was higher in the *coi1-16* mutants than that in the WT (Raya-Gonzalez et al., 2012). Furthermore, JA might act through the modulation of auxin accumulation and transport, as nicely demonstrated through the identification of the *Arabidopsis* mutant *jasmonate-induced defective lateral root1 (jdl1)* that repressed rather than induced the LRP initiation in the presence of MeJA, as observed in WT plants (Sun et al., 2009). Molecular analysis revealed that *JDL1* encodes an ANTHRANILATE SYNTHASE α1 (ASA1) that is a rate-limiting enzyme for the biosynthesis of Trp (Ljung et al., 2005; Stepanova et al., 2005).

Moreover, MeJA activated the transcriptional expression of *ASA1*, leading to the induction of IAA biosynthesis (Sun et al., 2009). Additionally, exogenous MeJA inhibited *PIN1* and *PIN2* expression at the protein level, thus negatively affecting the PAT-mediated auxin accumulation in the root basal meristem zone (Sun et al., 2009). The final combinational effect of MeJA on auxin biosynthesis and transport would result in increasing the local auxin accumulation in the basal meristem required for LR development. Recently, this observation was further confirmed by the JA induction of stages I-V LRP in WT seedlings and rather less at later stages, suggesting that JA affected LR development mainly at the initiation level (Raya-Gonzalez et al., 2012).

3. Strigolactone biology and crosstalk with other plant hormones in shaping root architecture

SLs are metabolites that are derived from carotenoids and act as important rhizosphere molecules as well as endogenous plant hormones. Strigol was the first natural SL identified as a germination stimulant of the root parasitic plant *Striga lutea* (Cook et al., 1966). Since then, many other SLs have been discovered from different plant species. They are all composed of four rings, designated A to D rings. The ABC rings carry one lactone group to which the D-ring is connected via an enol ether bridge. The part consisting of the C-D ring is highly conserved among SLs and is essential for bioactivity, whereas the A and B rings can have various modifications (Mangnus et al., 1992; Boyer et al., 2012; Umehara et al., 2015).

SLs have different functions in the rhizosphere and their first role detected was stimulation pf parasitic seed germination. Various SLs have been described in root exudates and a reduction in these components in the exudate results in a decreased *Striga* germination and attachment to the host root (Yoneyama et al., 2013). In addition, SLs also play a role in the interaction of plants

with arbuscular mycorrhizal fungi, on which SLs induce hyphal branching to promote the symbiotic interaction (Besserer et al., 2006; Parniske, 2008). SLs are produced throughout the whole plant, but mainly in the root (Ruyter-Spira et al., 2013). As endogenous hormones, SLs were first discovered to control shoot branching and, in the meantime, many other functions have been characterized (reviewed by Ruyter-Spira et al., 2013), such as stimulating secondary stem growth, inhibiting adventitious rooting, and affecting primary root growth, LR development, and root hair elongation (Fig 11). In all these functions, a crosstalk between SLs and other phytohormones seems to play a pivotal role (Cheng et al., 2013).

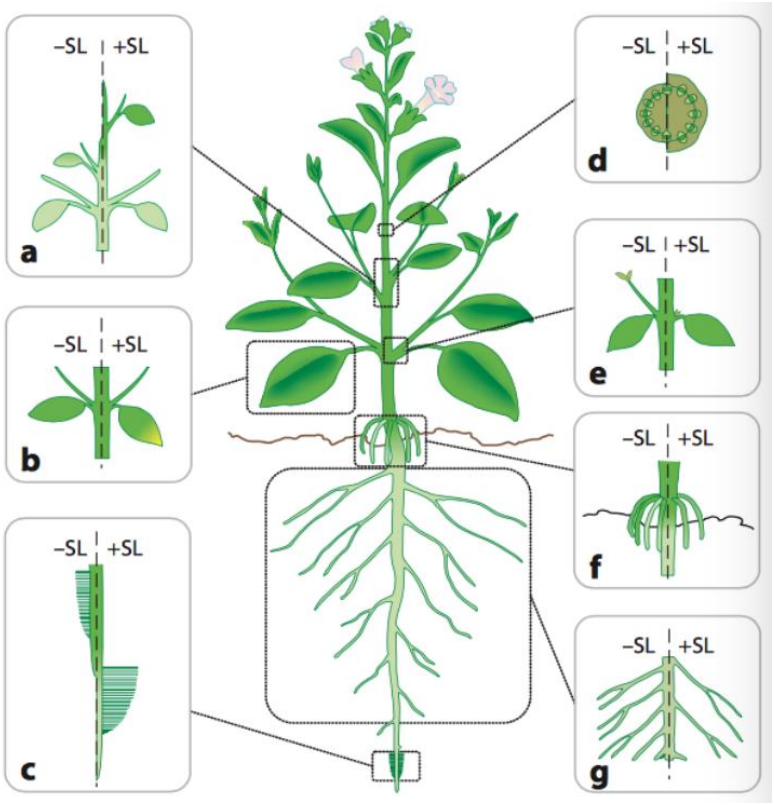

Figure 11. Roles of SLs in plant development. a. Stimulation of internode growth; b. acceleration of leaf senescence; c. enhancement of root hair elongation and primary root growth; d. induction of secondary growth; e. inhibition of axillary bud outgrowth; f and g. inhibition of adventitious root and LR formation, respectively. Picture was taken from Al-Babili and Bouwmeester, 2015.

3.1 Strigolactone biosynthesis

Pea, *Arabidopsis*, petunia (*Petunia hybrida*), and rice mutants affected in carotenoid metabolism displayed low or undetected SL levels and carotenoid biosynthesis inhibitors reduced the SL content, indicating that SLs are derived from carotenoids (Gomez-Roldan et al., 2008; Umehara et al., 2008). Further genetic studies, based on the branching phenotypes of these SL mutants, confirmed this hypothesis. In summary, four genes have been characterized that encode proteins involved in SL biosynthesis. They are *D27*, *CCD7*, *CCD8*, and *MAX1*, and the branching-related mutants affected in these genes are designated *ramosus* (*rms*) in pea (Beveridge et al., 1994, 1996, 2000), *more axillary growth* (*max*) in *Arabidopsis* (Stirnberg et al., 2002; Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2004; Booker et al., 2005; Crawford et al., 2010), *decreased apical dominance* in petunia (Snowden et al., 2005; Simons et al., 2007), and *dwarf* (*d*) or *high-tillering dwarf* (*htd*) in rice (Zou et al., 2006; Arite et al., 2007; Lin et al., 2009) (Table 1)

Table 1 Genes involved in strigolactone biosynthesis

Protein identity/ function	Gene name			
	Arabidopsis	**Pea**	**Petunia**	**Rice**
9-*cis/all-trans-β*-Carotene isomerase	*AtD27*			*D27*
Carotenoid cleavage dioxygenase 7 (CCD7)	*MAX3*	*RMS5*	*DAD3*	*HTD1/D17*
Carotenoid cleavage dioxygenase 8 (CCD8)	*MAX4*	*RMS1*	*DAD1*	*D10*
Cytochrome P450, cytochrome 711 (CYP711)	*MAX1*		*PbMAX 1*	Carlactone oxidase (Os01g0700900), Orobanchol synthase (Os01g0701400), Os01g0701500, Os02g0221900, Os06g0565100

Further biochemical studies revealed new insights into the SL biosynthesis pathway (Fig. 12). SLs are derived from all-*trans*-β-carotene and, initially, three enzymes, D27, CCD7, and CCD8 were involved in using all-*trans*-β-carotene as a substrate to produce an SL-biosynthetic intermediate, designated carlactone (CL) (Alder et al., 2012). CL has an SL-like carbon skeleton and an SL-like biological activity, such as stimulating parasite seed germination and rescuing the high-tillering phenotype of the rice SL mutants *d10* and *d27* affected in genes encoding the CCD8 and D27 enzymes, respectively (Alder et al., 2012; Seto et al., 2014). *In vitro* incubation of all-*trans*- and 9-*cis*-β-carotene with purified D27 showed that D27 catalyzes the isomerization of all-*trans*-β-carotene at the C9-C10 double-bond position, producing 9-*cis*-β-carotene (Alder et al., 2012). Although initially all-*trans*-β-carotene (C40) had been suggested to be the substrate of CCD7 to yield all-*trans*-β-apo-10−carotenal (C27), which is then cleaved by CCD8 into the C18 ketone β-apo-13-carotenone, a recent study showed that CCD7 is stereospecific for the 9-*cis*

configuration and thus uses the D27 product, 9-*cis*-β-carotene as the preferential substrate (Schwartz et al., 2004, Alder et al., 2012, reviewed by Ruyter-Spira et al., 2013). Because incubation of thioredoxin-CCD8 with 9-*cis*-β-carotene and all-*trans*-β-carotene yielded different products and only the incubation of CCD8 with 9-*cis*-β-carotene produced CL, CCD8 was proposed to use the substrate of CCD7 for SL biosynthesis (Bruno et al., 2014). Very recently, CL has been identified in rice and *Arabidopsis* and analysis of the absolute stereochemistry of CL demonstrated that endogenous CL has a (11*R*) configuration similar to that of the endogenous SL (-)-2'-*epi*-5DS (Seto et al., 2014). Previous grafting experiments with *max1*, *max3*, and *max4* mutants indicated that MAX1 is a component downstream of MAX3 and MAX4 and quantitative analysis of endogenous (*R*)-carlactone in *max1* and *max4* mutants in *Arabidopsis* roots suggested that (*R*)-CL was the substrate for MAX1 (Booker et al., 2005; Seto et al., 2014). Furthermore, feeding experiments of stable isotope-labeled (*R*)-CL [(*R*)-13C-CL] to the SL-deficient mutants *max4* and *max1max4* further confirmed this MAX1-dependent manner of the CL metabolism (Scaffidi et al., 2013). Recently, *Arabidopsis* MAX1 has been found to catalyze CL oxidations at C-19 to convert the C-19 methyl group into carboxylic acid, 9-desmethyl-9-carboxy-CL, designated carlactonoic acid (CLA), that is then converted into *ent*-2'-*epi*-5-dexoystrigol by an currently unknown enzyme or to methyl CLA (MeCLA) that is proposed to be biologically active in inhibiting shoot branching (Abe et al., 2014). Thus, the SL pathway seems to produce, besides SLs, other related molecules. Moreover, the CL-to-SL reaction is not a one-step reaction in rice (Zhang et al., 2014). One rice homolog of *Arabidopsis* MAX1, Os900, catalyzes CL into *ent*-2'-*epi*-5-deoxystrigol, whereas another rice homolog, Os1400, hydroxylates *ent*-2'-*epi*-5-deoxystrigol at C4 into orobachol. Rice carries five MAX1 genes, whereas, in contrast, only one *MAX1* gene exists in *Arabidopsis* (Booker et al., 2005; Zhang et al., 2014).

In rice, *CCD7 (HTD1)* and *CCD8 (D10)* are expressed throughout the vascular parenchyma cells of roots. In *Arabidopsis*, the expression of *MAX4* is localized only in the columella cap of the primary root and LRs, whereas *MAX1* is expressed throughout in the vascular root tissue (Bainbridge et al., 2005; Booker et al., 2005). Together with the grafting experiments with *max1* and *max4*, these experiments indicate that CL might be mobile and systemically spread before it is further metabolized by MAX1. The discovery of both SLs and CL in the xylem sap of *Arabidopsis* and tomato demonstrates that root-derived SLs are transported to the shoot via the xylem (Kohlen et al., 2011, 2012).

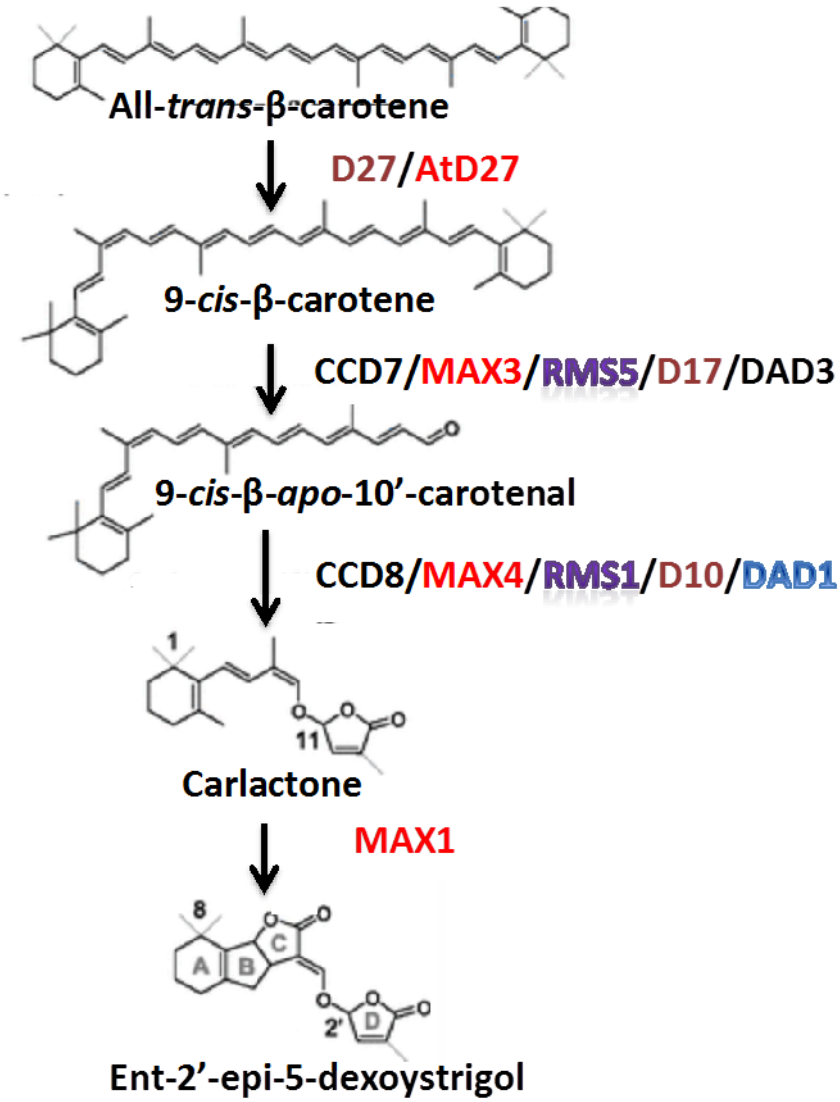

Figure 12. Route of SL biosynthesis. The key enzymes are DWARF27 (D27), CAROTENOID CLEAVAGE DIOXYGENASE7 (CCD7) and CCD8, MORE AXILLARY GROWTH1 (MAX1). AtD27 is homologous of D27 in *Arabidopsis*, MAX3/RAMOSUS5 (RMS5)/D17/DECREASED APICAL DOMINANCE3 (DAD3) and MAX4/RMS1/D10/DAD1 represent CCD7 and CCD8 respectively in *Arabidopsis*/pea/rice/petunia. Figure adapted from Seto and Yamaguchi, 2014.

3.2 The strigolactone signaling pathway

Genetic analyses, mainly on the branching phenotype of mutants, and the inability of the SL analog GR24 or natural SLs to recover the phenotypes revealed several genes involved in SL signaling (Fig 13).

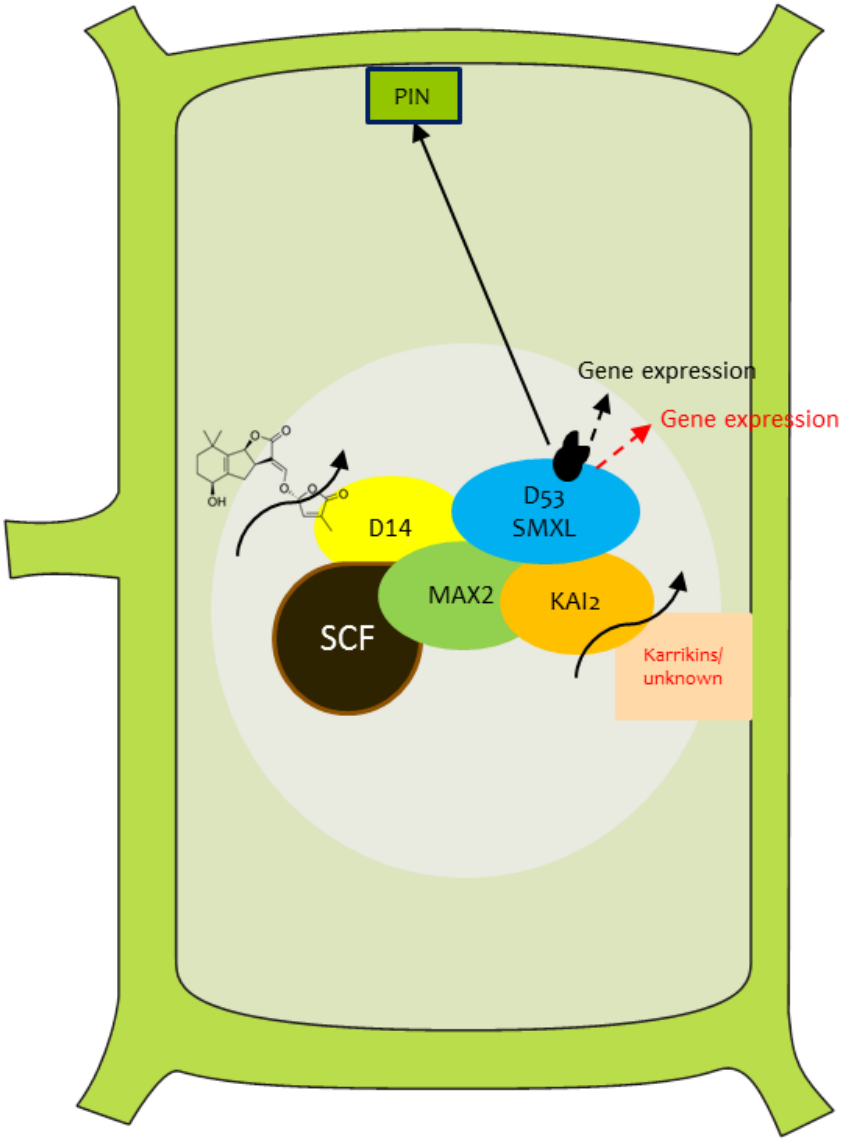

Figure 13. Model of (SL and karrikin signaling pathways. In the SL signaling pathway, DWARF14 (D14) binds to and hydrolyses SLs, activating the SCF^{MAX2} complex to ubiquinate D53/SMXL for proteasomal degradation. This signaling would further activate downstream responses such as PIN removal from the membrane and or diferential gene expression. In the karrikin signaling pathway, karrikin binds to a D14-like protein, KARRIKIN-INSENSITIVE 2 (KAI2), then uses a similar downstream signaling pathway as SL to activate signaling.

In rice, *d14* mutants were shown to be insensitive to the exogenous SL, whereas the accumulation of endogenous SL levels was high (Arite et al., 2009; Beveridge and Kyozuka 2010). Structure analysis together with biochemical approaches revealed that the D14-encoded protein was able to hydrolyze SLs and that the Ser-Asp-His catalytic triad was essential for SL signaling (Nakamura et al., 2013). A report on DAD2, an ortholog of the rice D14 from petunia, showed that DAD2 has a canonical catalytic triad with an internal cavity capable of accommodating SLs (Hamiaux et al., 2012). Moreover, D14 belongs to the α/β hydrolase family just like the gibberellin receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1) with the only difference that GID1 loses its capability to hydrolyze the substrate (Ueguchi-Tanaka et al., 2005). Hence, D14/DAD2 is supposed to act SL receptor.

The second gene essential for SL signaling is the gene encoding the F-box protein MAX2/RMS4/D3 (Beveridge et al., 1996; Ishikawa et al., 2005; Stirnberg et al., 2007; Drummond et al., 2012). MAX2 is an F-box protein containing leucine-rich repeats that is mainly localized in the nucleus and has been shown to be part of an SCF^{MAX2} complex (Stirnberg et al., 2007). This protein belongs to the same F-box protein clade as the COI1 receptor of JAs and the TIR receptors of auxins (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Sheard et al., 2010). Hence, this protein is expected to ubiquitinate specific proteins to target them for proteasomal degradation. In rice and petunia, yeast two-hybrid analysis and immunoprecipitation assays have shown that D14/DAD2 and D3/MAX2 interact with each other in a SL-dependent manner (Hamiaux et al., 2012; Jiang et al., 2013; Zhou et al., 2013). Moreover, in petunia, the catalytic triad is required for the yeast two-hybrid interaction of DAD2 with MAX2 and the interaction of DAD2 and MAX2 results in hydrolysis of GR24 by DAD2 (Hamiaux et al., 2012).

Recently several possible candidates for MAX2 targets have been proposed (Jiang et al., 2013; Zhou et al., 2013; Stanga et al., 2013; Wang et al., 2013; Soundappan et al., 2015; Wang et al., 2015). The characterization of the dominant SL-insensitive *dwarf 53* (*d53*) mutant of rice resulted in the identification of the D53 protein as a substrate of SCF^{MAX2}. Addition of GR24 led to the degradation of D53 and this process required both D14 and MAX2 (Jiang et al., 2013; Zhou et al., 2013). *D53* encodes a protein belonging to the double Clp-N motif-containing P-loop nucleoside triphosphate hydrolase superfamily and might function as a repressor of SL signaling, because D53 interacts with TPL/TPL-related (TPR) proteins that are known corepressors in various plant hormone signaling and plant development pathways (Jiang et al., 2013). Based on these data, SLs have been proposed to bind to D14, once they have entered the cell, whereafter the D14-SL complex is recognized by SCF^{MAX2}, resulting in SL hydrolysis and in D53 ubiquitination and subsequent degradation by the 26S proteasome. As a consequence, SL-responsive genes would be expressed (Jiang et al., 2013; Zhou et al., 2013). In agreement with the studies in rice, a suppressor screen of the *Arabidopsis max2* mutant resulted in the identification of *SUPPRESSOR OF MAX2 1* (*SMAX1)*, a gene encoding a homolog of *D53* (Stanga et al., 2013). Very recently, three other SMAX1-LIKE (SMXL) proteins, SMXL6, SMXL7 and SMXL8, have been demonstrated to be degraded in a SL and MAX2-dependent manner (Soundappan et al., 2015; Wang et al., 2015). In addition, D14 and SL have been reported to stimulate together the MAX2-dependent ubiquitination and degradation of the transcriptional regulator brassinolide-insensitive1-EMS-suppressor1 (BES1), which is a key component of brassinosteroid signaling in *Arabidopsis* (Wang et al., 2013). Simultaneously, D14 had also been suggested to interact with a gibberellin signaling repressor SLENDER 1 (SLR1) in a SL-dependent manner (Nakamura et al., 2013).

The genetic analysis of the SL mutants revealed that the *Arabidopsis max2* mutants carried more phenotypes than the biosynthesis mutants (Stirnberg et al., 2002; Shen et al., 2012; Water and Smith, 2013). For instance, *max2* mutants show a enlarged hypocotyl but not the SL biosynthesis mutants and the *Arabidopsis d14* mutant (Scaffidi et al., 2013), indicative that MAX2 is involved in the signaling pathway of still other compounds (Waters et al., 2012). Indeed, karrikins, SL-analogous compounds from forest-fire smoke also use MAX2 to induce seed germination (Nelson et al., 2012). Furthermore, a mutation in the *KARRIKIN-INSENSITIVE 2* (*KAI2*) (also known as D14-like) genee encoding a paralog of *D14*, was evenly affected in karrikin-induced germination (Waters et al., 2012). Hence, SLs and karrikins are perceived by two related proteins, whereafter the signaling converts to the SCF^{MAX2} complex (Nelson et al., 2011; Waters et al., 2013). As *kai2* has many MAX2-overlapping phenotypes that are absent in the *d14* mutant, KAI2 is expected to also perceive endogenous SL and karrikin related molecules to regulate seed germination, seedling growth, and leaf and rosette development (Waters et al., 2012; Soundappan et al., 2015). The SMAX1 protein is believed to be degraded by the SCF^{MAX2} complex downstream of the KAI2 signaling (Stanga et al., 2013). Moreover, the commonly used GR24 as a synthetic SL has been shown to be able to mimic both signaling related to D14 and KAI2 (Zhao et al., 2013).

3.3 The function of strigolactone in root architecture

Since the discovery of SLs as endogenous hormones involved in the regulation of shoot branching, many other functions in different plant developmental processes have been described, including the regulation of the root system architecture (RSA) (reviewed by Waldie et al., 2014; Kapulnik and Koltai, 2014; Koltai, 2015). Feeding experiments with GR24 on plants showed that GR24 repressed LR development in WT and the SL-biosynthesis mutants (*max3* and *max4*),

but not in the SL-signaling *max2* mutants (Kapulnik et al., 2011a). In agreement, the SL-signaling mutant *max2* in *Arabidopsis* has more lateral roots than WT (Kapulnik et al., 2011a). Staging experiments further showed that application of GR24 reduced LRI as well as LR outgrowth, indicating that SLs are negative regulators in LR development, especially during LRI and LR elongation in a MAX2-dependent manner (Ruyter-Spira et al., 2011). Interestingly, whereas under sufficient inorganic phosphate (Pi) growth conditions, application of GR24 resulted in a reduction in LR formation, GR24 application led to increased LR formation under Pi-limiting growth conditions (Mayzlish-Gati et al., 2012). This effect has been hypothesized to be due to the modified auxin landscape provoked by changing Pi conditions (Mayzlish-Gati et al., 2012).

SLs also affect LR development in rice and *Medicago truncatula* (Sun et al., 2014; De Cuyper et al., 2015). In rice, under Pi- and N-limiting conditions, GR24 application restored the LRD in WT, and *d10* and *d27* mutants, but not in the *d3* mutants. Further studies indicated that SLs are induced by nutrient-limiting conditions and affect LR development via D3 in rice. In *M. truncatula,* GR24 application has an inhibitory effect on the lateral rooting. In addition, treatment with GR24 of plants inoculated with *Sinorhizobium meliloti* affected the nodule number both positively and negatively, depending on the applied concentration.

In addition to affecting LR development, SLs also regulate primary root growth (Ruyter-Spira et al., 2011). GR24 application led to primary root elongation and an increase in meristem cell numbers under sufficient Pi and sugar conditions, whereas, with increasing GR24 doses, an opposite effect – inhibition of the primary root length – was observed (Ruyter-Spira et al., 2011). Under carbohydrate-limitating conditions that lead to a reduction in primary root length, a positive effect on the primary root elongation and meristem cell numbers was observed with

GR24 treatment at all different concentrations (Ruyter-Spira et al., 2011). Consistently, the primary root length of *Arabidopsis* mutants deficient in SL biosynthesis and signaling was shorter than the WT under carbohydrate-limitating conditions (Ruyter-Spira et al., 2011). Hence, SLs are proposed to be positive regulators in primary root elongation, but their impact to depend on the growth conditions (Ruyter-Spira et al., 2011). Importantly, the expression of *MAX2*, driven by the *SCARECROW* (*SCR*) promoter, was sufficient to confer a response to GR24 in a *max2* mutant background for both LR formation and cell numbers of the main root meristem (Koren et al., 2013). *SCR* is expressed in the root endodermis and quiescence center suggesting that the endodermis is important in SL regulation of LR and primary root growth (Koren et al., 2013).

As part of the root architecture, adventitious root growth is also regulated by SLs. The SL-deficient and response mutants in *Arabidopsis* and pea showed an enhanced adventitious root formation and, in agreement with the LR results, application of the SL analog GR24 suppressed adventitious root formation (Rasmussen et al., 2012a, 2012b). By means of the *pCYCB1:GUS* reporter line, an early marker for the initiation of adventitious root primordia, SLs restrain the number of adventitious roots by inhibiting the first formative cell divisions of the founder cells, because the number of sites expressing *CYCB1:GUS* was significantly higher in the *max2* mutants than in the WT (Rasmussen et al., 2012b). This phenotype was also observed in tomato (Kohlen et al., 2012). Very recently, SLs were reported to positively regulate adventitious root production in rice (Sun et al., 2015). In rice, the SL mutants with impaired SL biosynthesis and signaling displayed a reduced adventitious root growth and GR24 application in SL-biosynthesis mutants increased the number of adventitious root. Thus, the effect of SLs on adventitious root development might depend on the species.

Another role of SLs in the RSA is the regulation of root hair (RH) elongation. In tomato, high doses of exogenous GR24 led to shorter and fewer RHs than in the control (Koltai et al., 2010). However, exposing *Arabidopsis* WT and SL-deficient mutants (*max3* and *max4*) to GR24 resulted in a significant increase in RH length, whereas this effect was not observed in the SL-signaling mutants (*max2*), suggesting that the SL effect on RH development is positive and mediated via MAX2 (Kapulnik et al., 2011a).

In conclusion, we can deduce that SLs might regulate root architecture depending on the external environmental conditions and that SLs are likely to play a role together with other plant hormones to regulate the RSA.

3.4 The strigolactone and auxin interplay.

The interplay of SL and auxin in shoot branching has been well studied (Hayward et al., 2009; Ferguson et al., 2009; Domagalska and Leyser, 2011; Shinohara et al., 2013). SL addition leads to a reduction in PIN1 levels in xylem parenchyma cells, accompanied by a reduction in PAT (Crawford et al., 2010). Consistently, high levels of branching together with high levels of PIN1 and PAT and high auxin concentrations in the main stem of SL biosynthesis mutants can be observed (Bennett et al., 2006). To explain how SLs act with auxin to inhibit axillary bud growth, a computer modeling study was performed in which different processes affecting PAT were included (Prusinkiewicz et al., 2009). This study revealed that SLs act to reduce the accumulation of PIN1 on the plasma membrane, leading to the auxin transport canalization that carries auxin away from the bud apex and establishes vascular connectivity between the bud and the remainder the plant that is more difficult to achieve (Prusinkiewicz et al., 2009). The interruption of the auxin transport canalization finally results in SL-mediated bud inhibition.

Another recent computer modeling work provided additional support for the canalization-based model for shoot branching control by using three *Arabidopsis* mutants, *max2*, *gnom*, and *tir3* (Shinohara et al., 2013) and showed that SL signaling was stimulated to increase the removal rate of PIN1 from the plasma membrane of stem parenchyma cells (Shinohara et al., 2013). Conversely, auxin also regulates SL production, via stimulation of the transcription of *MAX3* and *MAX4*. Consistently, removal of the apical auxin source leads to a strong decrease in SL-biosynthetic gene expression (Foo et al., 2005; Hayward et al., 2009).

Also to control root architecture, an interplay with auxin has been proposed (reviewed by Cheng et al., 2013; Koltai, 2015; Al-Babili and Bouwmeester, 2015). It is well-known that LRI depends on the local accumulation of auxin in root pericycle cells adjacent to the xylem vessels (Casimiro et al., 2001; Benková et al., 2003; Dubrovsky et al., 2008). In contrast, LR development in young roots is supported by shoot-derived auxin that is then delivered into LRP via the PAT and imported into the developing LRP via PIN2 repolarization (Pandya-Kumar et al., 2014). Treatment with GR24 could not remove PIN1 from the plasma membrane of the root cells as opposed to the observation in the shoot (Shinohara et al., 2013). On the contrary, long exposures to GR24 reduced the levels of PIN1, PIN3, and PIN7 in the root apical meristem (Ruyter-Spira et al., 2011). As SLs have been shown to reduce the auxin levels in the aerial plant parts, probably not enough auxin could reach the root to sustain LRP development (Ruyter-Spira et al., 2011). Hence, based on this information, the impact of SL on LR development might occur by affecting the PIN1 accumulation on the plasma membranes of stem parenchyma cells (Prusinkiewicz et al., 2009; Shinohara et al., 2013).

As far as the SL influence on the primary root growth is concerned, the regulatory role of SLs in primary root growth might be mediated via the inhibitory effect on the auxin efflux carriers

(Koltai et al., 2010; Ruyter-Spira et al., 2011; Koren et al., 2013). Accordingly, the intensities of PIN1, PIN3, and PIN7-GFP decreased in the provascular tissues of primary root tips under GR24 treatment and both endogenous SLs and GR24 were able to reduce the basipetal auxin transport in the root (Ruyter-Spira et al., 2011). Intriguingly, *CCD8* in *Arabidopsis* is specifically expressed in the cortical and epidermal cells of the transition zone upon auxin treatment and the expression of the SL-signaling gene *MAX2* is also elevated in this root zone (Foo et al., 2005; Arite et al., 2007; Hayward et al., 2009). Thus, these data would indicate that the SL signaling might lead to a reduction in PIN protein accumulation, leading to an increase in the transition zone size (Ruyter-Spira et al., 2011). In addition, high doses of exogenous SLs cause distortion of the linear root length that is accompanied with asymmetric cell length, probably caused by asymmetric auxin accumulation in the elongation zone of the root via asymmetric PIN3 distribution (Ruyter-Spira et al., 2011). In contrast, auxin has been shown to induce SL synthesis in the root by inducing *CCD7* and *CCD8* expression, suggesting a feedback regulation between auxin and SLs (Hayward et al., 2009).

As far as RH development is concerned, SL signaling is not necessary for the auxin-induced RH elongation, because *max2* mutants were as responsive to auxin as the WT (Kapulnik et al., 2011b). On the contratry, the auxin receptor mutant *tir1-1* displayed a reduced response to GR24, indicating that auxin perception is required for the SL response on RH elongation (Kapulnik et al., 2011b). However, because *tir1-1* also showed a reduced sensitivity to 1-aminocyclopropane-1-carboxylate (ACC) and because of the important role of ethylene (ET) signaling in the RH response (see below), this decreased sensitivity of *tir1-1* to GR24 has been proposed to result from its reduced response to ET (Kapulnik et al., 2011b).

In the regulation of adventitious roots, the interplay between SLs and auxins appears to be more complex. Auxin application promotes the expression of SL synthesis genes in plant stems (Foo et al., 2005; Hayward et al., 2009), possibly the reason for the negative regulation of auxin on adventitious rooting. Nevertheless, this auxin-induced SL signaling is not the key process that affects adventitious rooting, because both SL and auxin mutants respond to auxin and SL treatments, respectively (Rasmussen et al., 2012b). There are two main hypotheses to explain the interplay of SL and auxin in adventitious rooting. One is that SL signaling affects the auxin sensitivity of tissues and the other is that SLs may reduce the amount of locally available free auxin, thereby inducing adventitious rooting (Kohlen et al., 2012; Rasmussen et al., 2012b).

3.5 The strigolactone-cytokinin crosstalk

Similar to the interplay of SLs and auxins, the crosstalk between SLs and CKs has been mainly studied in the context of shoot branching. CKs are known to play a stimulatory role in axillary branching in the shoot. By means of SL mutants and exogenous CKs, CKs and SLs have been suggested to act antagonistically in the regulation of bud outgrowth (Dun et al., 2012). This antagonism would act at the expression level of their common bud-specific targets. The expression of *BRANCHED1* (*BRC1)* and its homolog *BRC2*, is largely specific to axillary meristems and buds (Aguilar-Martinez et al., 2007). *BRC1* has been shown to be upregulated by GR24 and downregulated by CK (Dun et al., 2012). Furthermore, SLs might feed back on CK production by inhibiting *IPT1* expression (Dun et al., 2012).

Generally, to control the root architecture, auxin plays a fundamental role, whereas the other phytohormones modulate auxin action to affect the root architecture, as is also the case for CKs. In contrast to auxin, exogenous CKs suppress LR formation and primary root growth and

transgenic *Arabidopsis* plants with decreased CK levels display increased root branching and enhanced primary root growth (Werner et al. 2003). These negative CK effects on the root architecture have also been confirmed by the study of CK perception and signaling mutants that display a fast-growing primary root and increased LR branching (Riefler et al. 2006). As far as LR development is concerned, CKs interfere with the initial asymmetric division for a LR through disrupting the expression of *PIN1/PIN2/PIN3/PIN7* in the LR founder cells, perturbing the establishment of an auxin gradient that disturbs the LR formation (Laplaze et al., 2007). Recently, SHY2, a key component in the auxin-CK crosstalk for the regulation of meristem differentiation (Dello Ioio et al., 2008; Perilli et al., 2012) might be involved in endodermal MAX2-mediated SL signaling to regulate meristem size and LR development (Koren et al., 2013), but further insights into the SL-CK crosstalk are not available. Because SHY2 was shown to regulate *PIN* gene expression (Dello Ioio et al., 2008), the SL insensitivity of the *shy2* loss-of-function mutant might be due to an impact on the auxin transport capacity (Koren et al., 2013).

SLs and CKs appear to act independently to suppress adventitious rooting (Rasmussen et al., 2012b; Rasmussen et al., 2013), because CK treatment of the SL-deficient and response mutants repressed adventitious rooting to an extent similar to that observed in CK-treated WT plants. CK biosynthesis and signaling mutants also responded to exogenous SLs similarly as WT plants (Rasmussen et al., 2012b).

3.6 The interactions of strigolactone-ethylene and strigolactone-jasmonate

Currently, there are no data available on the interplay between SLs and JAs in root development. As described above, JAs play a positive role in LR development, whereas they negatively

regulate primary root growth, indicating that these two hormones might act antagonistically to control RSA.

The interaction between SL and ET has only been studied in the control of RH elongation and the response to biotic factors. ET synthesis in response to stress inhibits organ growth (Achard et al., 2006), whereas it stimulates adventitious root formation in wetland plants in response to flooding (Visser et al., 1996; Lorbiecke and Sauter, 1999; Steffens et al., 2006). ET has been shown to trigger auxin biosynthesis and basipetal auxin transport toward the elongation zone of the root meristem, where it contributes to a cell elongation-inhibiting local auxin response (Stepanova et al., 2005). This ET-dependent inhibition of primary root growth is probably mediated by *WEAK ETHYLENE INSENSITIVE2* (*WEI2*) (also designated as *ASA1*) and *WEI7* (*ASB1*) (Stepanova et al., 2005). In addition, auxin signaling pathways are also required for ET inhibition of root elongation. Mutants with stabilized AUXIN RESISTANT2 (AXR2)/IAA7 and AXR3/IAA17 proteins show ET-resistant root growth, whereas the mutants bearing stabilized SHY2/IAA3 and SLR/IAA14 are strongly resistant to auxin, but not to ET (Růžička et al., 2007; Swarup et al., 2007). The negative role of ET in LR development was implied from enhanced PAT, thus preventing the localized accumulation of auxin needed to induce LR formation (Ivanchenko et al., 2008; Lewis et al., 2011). As SLs and ET have been shown to have a positive effect on RH elongation and because the ET-signaling mutants *ein2* and *etr1* have a reduced sensitivity to GR24, ET signaling might mediate the GR24 effect on RH elongation (Kapulnik et al., 2011b). In contrast, the SL signaling mutant *max2* was as sensitive to the ET precursor ACC as thee WT, indicating that SL signaling is not necessary for the ET response. Morevover, because ET synthesis is associated with increased *ACC Synthase2* (*ACS2)* gene expression and application of GR24 significantly increased *ACS2* expression, the effect of SLs on RH elongation has been proposed to involve ET biosynthesis (Kapulnik et al., 2011b).

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Chapter II

Strigolactones spatially influence lateral root development through interplaying with cytokinin signaling

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ABSTRACT

Strigolactones (SLs) are important rhizosphere signals that act as phytohormones with also multiple functions in the root, such as the modulation of lateral root (LR) development. Here, we show that treatment with the SL analog GR24 does not affect lateral root initiation but negatively influenced lateral root priming as well as the emergence of LRs, the latter especially near the root-shoot junction. We investigated the interaction of GR24 with cytokinins for these effects and have found that the cytokinin module *ARABIDOPSIS HISTIDINE KINASE3* (*AHK3*)/*ARABIDOPSIS RESPONSE REGULATOR1* (*ARR1*)/*ARR12* interacts with the GR24-dependent reduction in LR development, because mutants in this pathway rendered LR development insensitive to GR24. Additionally, pharmacological analysis, mutant analysis as well as gene expression analysis indicated that the affected polar auxin transport stream in mutants of the *AHK3-ARR1ARR12* module could be the underlying cause of their GR24 insensitivity. Altogether, the data reveal that in the root the GR24 effect on LR development depends on the hormonal landscape, which results from the intimate connection with auxins and cytokinins, two main players in LR development.

INTRODUCTION

Strigolactones (SLs) are phytohormones that affect shoot lateral branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008) and many other processes, such as photomorphogenesis, drought tolerance, leaf senescence, and secondary growth, among others (Woo *et al*., 2001; Snowden *et al*., 2005; Shen *et al*., 2007, 2012; Tsuchiya *et al*., 2010; Agusti *et al*., 2011; Bu *et al*., 2014). In the rhizosphere, SLs influence interactions of the host plant with neighboring organisms, such as

root-parasitic plants, mycorrrhizal fungi, and rhizobia (for review, see Xie *et al*., 2010; Rasmussen *et al*., 2013). The root system architecture itself is also affected by SLs, because SLs influence adventitious root development, main root growth, root hair development, and lateral root (LR) development (Kapulnik *et al*., 2011a, 2011b; Ruyter-Spira *et al*., 2011; Mayzlish-Gati *et al*., 2012; Rasmussen *et al*., 2012, 2013; Sun *et al*., 2014). LR development follows a tightly regulated program, consisting of several successive steps (reviewed by Péret *et al.*, 2009). The first step consists of lateral root priming, which occurs in the xylem pole pericycle (XPP) cells of the basal meristem zone of the main root tip. As the root grows, the primed XPP cells enter the elongation zone, where the first asymmetric cell division of the primed XPP cells takes place. This leads to the formation of a lateral root primordium (LRP), a process which is called LR initiation. Then subsequent rounds of controlled cell divisions give rise to an outgrowth of a typical dome-shaped primordium and finally an emerged lateral root (LR).

Regarding LR development, addition of the SL analog GR24 was found to reduce the LR density (LRD), because of a diminished LR initiation and LR outgrowth (Koltai *et al.*, 2010; Kapulnik *et al.*, 2011b; Ruyter-Spira *et al.*, 2011). In *Arabidopsis thaliana*, mutants in the F-box protein MORE AXILLARY GROWTH2 (MAX2) are perturbed in SL perception and display higher LRDs than the wild-type (WT) plants (Kapulnik *et al.*, 2011b; Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011). When MAX2 function was restored specifically in the root endodermis of *max2* mutants, their insensitivity could be partially complemented (Koren *et al.*, 2013). SLs are perceived by an α/β-hydrolase, DWARF14 (D14), that binds and hydrolyzes SLs and plays a central role in downstream signaling activation (Hamiaux *et al.*, 2012; Zhao *et al.*, 2013). In petunia (*Petunia hybrida*) and rice (*Oryza sativa*), D14 interacts with MAX2/D3, a nuclear-localized F-box protein that participates in the Skp-Cullin-F-box (SCF) complexes and, thus, can

mediate the ubiquitin-dependent degradation of signaling proteins (Hamiaux *et al.*, 2012; Zhao *et al.*, 2013).

The interaction of SLs with auxins and cytokinins in regulation of shoot lateral branching has been thoroughly studied mainly in pea (*Pisum sativum*) and *Arabidopsis* (for a review, see Stirnberg *et al*., 2010; Cheng *et al*., 2013; Rasmussen *et al*., 2013a). Indeed, SL biosynthesis and signaling are intimately connected with auxin transport regulation (Foo *et al*., 2005; Bennett *et al*., 2006; Brewer *et al*., 2009; Ferguson and Beveridge, 2009; Hayward *et al*., 2009; Crawford *et al*., 2010; Koltai *et al*., 2010; Shinohara *et al*., 2013; Pandya-Kumar *et al*., 2014). The application of GR24 reduces the basipetal auxin transport and the accumulation of PIN-FORMED1 (PIN1) in the plasma membrane of xylem parenchyma cells in the shoot in a MAX2-dependent manner (Crawford *et al*., 2010). Moreover, in buds, SLs promote PIN1 endocytosis through a clathrin-dependent mechanism that occurs independently of de novo protein synthesis (Shinohara *et al*., 2013). In pea, SLs have been demonstrated to act also independently of auxin (Brewer *et al*., 2015). Interestingly, SLs could inhibit shoot lateral branching only when a competing auxin source was available (Crawford *et al*., 2010; Liang *et al*., 2010). The auxin landscape also influences the SL control on branching, because the negative effect on shoot lateral branching disappeared and even became positive when the auxin homeostasis was changed (Shinohara *et al*., 2013). In buds, SLs and cytokinins are known to interact antagonistically and locally (Dun *et al*., 2012; Zhang *et al*., 2010; Hu *et al*., 2014), probably through their common target, BRANCHED1 (BRC1) in Arabidopsis (Minakuchi *et al*., 2010; Braun *et al*., 2012; Dun *et al*., 2012).

Also in the root, the interaction of SLs with auxins has been investigated. PIN1, PIN3, and PIN7 protein levels are reduced upon prolonged treatment with GR24 (Ruyter-Spira *et al*., 2011).

Additionally, during GR24-induced root hair elongation, PIN2 abundance increases at the apical plasma membrane of epidermal cells, suggesting that SLs affect PIN2 endocytosis and endosomal trafficking via actin dynamics in a MAX2-dependent manner (Pandya-Kumar *et al*., 2014). The inhibitory effect of GR24 on LR development can be reverted to an induction rather than a reduction of LRD by applying a high dose of auxin, or under low phosphate conditions that may increase the auxin sensitivity (Pérez-Torres *et al*., 2008; Ruyter-Spira *et al*., 2011). These observations suggest that, just as for branching, changes in the auxin landscape could modulate the impact of GR24 (Ruyter-Spira *et al*., 2011).

Cytokinins are also well known to influence the root architecture (reviewed in Vanstraelen and Benková, 2012). Cytokinin signaling negatively affects LR development by impinging on PIN-dependent auxin transport (Laplaze *et al*., 2007; Bishopp *et al*., 2011; Marhavý *et al*., 2011, 2014; Bielach *et al*., 2012; Chang *et al*., 2013; Moreira *et al*., 2013). Interaction of SLs with cytokinins during LR development has been poorly studied, but *max2-1* mutants have been reported to have a reduced sensitivity to the synthetic cytokinin 6-benzylaminopurine (BAP) (Koren *et al*., 2013).

Here, LR priming as well as outgrowth is shown to be modulated by treatment with GR24, the latter in a spatiotemporal way mainly affecting the emergence of the LRs, which are the closest to the root-shoot junction. In addition, the *ARABIDOPSIS HISTIDINE KINASE3* (*AHK3*)/*ARABIDOPSIS RESPONSE REGULATOR1* (*ARR1*)/*ARR12* cytokinin signaling module interacts with SLs to affect LR development, probably through changes in polar auxin transport. Altogether, the results put the SL action on LR development in the auxin landscape context via cross-talk mechanisms with cytokinin signaling.

RESULTS

GR24 reduces lateral rooting in Arabidopsis by affecting LR emergence, especially near the shoot-root-shoot junction in a MAX2-dependent manner

The overall MAX2-dependent reduction in LRD caused by GR24 application had already been reported (Kapulnik *et al.*, 2011b; Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011), but phenotypical insights into this event are still lacking. Upon GR24 treatment, the first emerged LR had an altered position and this effect was abolished in the *max2-1* mutant. When plants were grown without GR24 (mock), the distance from the hypocotyl to the first emerged LR was on average 3.37mm, whereas when grown in the presence of GR24 it increased to 6.27mm in WT plants (Fig. 1A).

To understand this effect, the LR development was spatiotemporally followed, with specific focus on the upper root zone. Therefore, the expression of the early LR marker GATA23 that indicates prebranch sites (De Rybel *et al.*, 2010) was used and combined with the staging of the LR primordia (Malamy and Benfey, 1997), in both WT and *max2-1* plants, under mock and GR24 treatments (Fig. 1E, F). As such, all sites in which an LR could develop were visualized from the root–shoot junction down to the root meristem at 4 DAG (Fig. 1E; Supplementary Fig. S1A). The progression in LR development was subsequently analyzed at 9 DAG (Fig. 1F; Supplementary Fig. S1B) to obtain a spatiotemporal view of how the LR primordium development was affected by GR24 treatment. Fewer GATA23-marked sites were observed at 9 DAG than at 4 DAG, implying that not all primed sites developed into an LR primordium. When the number of LR sites between mock and GR24-grown plants was compared, slightly, but significantly, fewer sites were counted upon GR24 treatment, both at 4 and 9 DAG (Fig. 1B),

indicating that GR24 treatment reduced the total number of prebranch sites in WT, but not in *max2-1*, seedlings (Fig. 1B). Concerning initiated patches (see Materials and Methods), mock and GR24-grown roots of both WT and *max2-1* seedlings did not differ, suggesting that GR24 had no effect on LR initiation, once the prebranch site had been formed (Fig. 1C). GR24 treatment also affected LR outgrowth (Kapulnik *et al.*, 2011b; Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011). When the percentage of emerged patches was calculated, significantly fewer sites were counted on GR24-grown roots than on control roots, but again not on *max2-1* roots (Fig. 1D). Interestingly, when the emergence pattern was analyzed at 9 DAG (Fig. 1F), the LR outgrowth inhibition was most pronounced at positions 1–8, corresponding to the LR primordia closest to the root–shoot junction, but did not occur in the *max2-1* mutant (see Supplementary Fig. S1B). These data indicate that mainly the first formed LR primordia, thus those near the root–shoot junction, do not develop when plants are grown in the presence of GR24 and that this effect depends on MAX2.

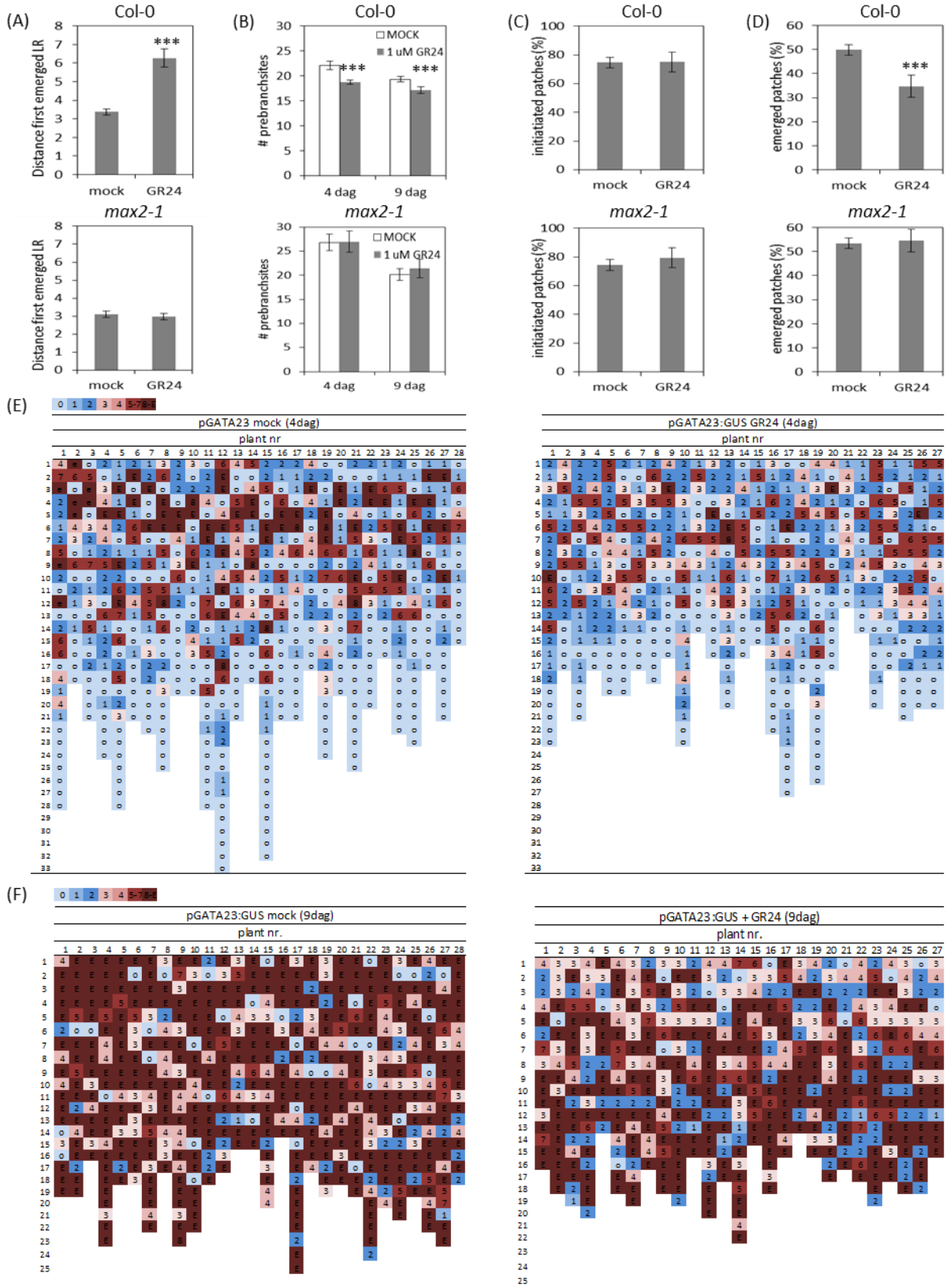

Figure 1. Effect of exogenous GR24 on LR development near the root-shoot junction.

(A) Distance to the first emerged LR in Col-0 (top) and max2-1 (bottom). (B) Total number of prebranch sites under mock (white bars) and GR24 treatment (gray bars), 4 and 9 DAG in Col-0 (top) and max2-1 (bottom). (C) Percentage of initiated patches under mock and 1 µM GR24 treatments in Col-0 (top) and max2-1 (bottom) at 9 DAG. (D) Percentage of emerged patches under mock and GR24 treatment in Col-0 (top) and max2-1 (bottom). (A–D) Data presented are means ± standard error (SE) of three biological repeats (n>20). ***P<0.001, according to the Student's t-test. (E, F) Stages of LR primordia via GATA23:GUS staining in Col-0 under mock (left) and GR24 treatment (right) at 4 DAG (E) and 9 DAG (F). All events, possibly leading to emerged LRs, were scored in individual plants, color-coded, and for each plant, vertically ordered from the closest to the hypocotyl (up) downward to the meristem (down). The root fragments used for analysis were comparable in length. Data of one representative experiment are shown. The experiments were repeated three times with similar results.

The cytokinin signaling components AHK3 and ARR1ARR12 mediate the effect of GR24 on LR development

Both cytokinins and SLs have been described as negative regulators of LR development in *Arabidopsis* (Benková *et al*., 2003; Li *et al*., 2006; Laplaze *et al*., 2007; Kapulnik *et al*., 2011b; Ruyter-Spira *et al*., 2011). Therefore, the link between the GR24-mediated LRD reduction and the cytokinin-mediated LRD inhibition was investigated in further detail. Firstly, the LRD of several cytokinin signaling mutants, single and higher-order mutants affected in the cytokinin receptors *CYTOKININ RESPONSE1 (CRE1)/AHK4*, *AHK2*, and/or *AHK3* (see Materials and Methods) was examined upon treatment with 1 µM GR24 (Fig. 2A, B). For all tested genotypes, GR24 treatment did not significantly affect the main root length (Supplementary Fig. S2). For Col-0, *cre1/ahk4*, and *ahk2*, the LRD was significantly reduced upon GR24 treatment, but not for the *ahk3* mutant (Fig. 2A). In the double cytokinin receptor mutant a*hk2ahk4*, the LRD

decreased significantly upon GR24 treatment, whereas no significant changes in LRD were observed for *ahk2ahk3* and *ahk3ahk4* between mock and GR24 treatment (Fig. 2B). Taken together, these data show that in mutants specifically affected in one member of the cytokinin receptor family, i.e. *AHK3* (*ahk3, ahk2ahk3*, and *ahk3ahk4*), the GR24 impact on LRD was abolished, whereas other cytokinin receptor mutants responded as WT plants. The *AHK3* expression pattern was unaffected by GR24 treatment (Supplementary Fig. S3).

These observations prompted the investigation of the downstream signaling components of the cytokinin perception machinery. As the B-type *ARR1* and *ARR12* are involved in mediating the *AHK3*-dependent effects in the root elongation zone (Dello Ioio *et al*., 2007, 2008), GR24 impact on the LRD was tested in mutants of these response regulators. The single mutants *arr1* and *arr12* displayed a sensitivity to GR24 similar to that of Col-0 (Fig. 2C), but the double mutant *arr1arr12* did not, indicating that both ARRs need to be disrupted to interfere with the GR24 effect on LR development (Fig. 2C).

Having established that *AHK3* and *ARR1ARR12* are involved in the GR24-mediated reduction of LRD, we analyzed whether a decrease in cytokinin response would affect the GR24-mediated LRD reduction. Therefore, we tested the sensitivity of higher-order A-type ARR mutants to GR24, because these negative regulators of the cytokinin response are known to act redundantly in root architecture control (To *et al*., 2004; Zhang *et al*., 2011). The *arr5/arr6/arr8/arr9* (*arr5689*) and *arr3/arr4/arr5/arr6/arr8/arr9* (*arr345689*) mutants showed a significant increase in sensitivity to GR24: LRD decreased by 37% in WT and by 58% and 67% in *arr5689* and *arr345689*, respectively (Fig. 2D). Hence, these data support the hypothesis that an altered cytokinin responsiveness can enhance (A-type ARR) or repress (B-type ARR or AHK3) the

GR24 effect on LR development. Taken together, these experiments demonstrate that specific cytokinin signaling components are needed for the GR24 action on LR development.

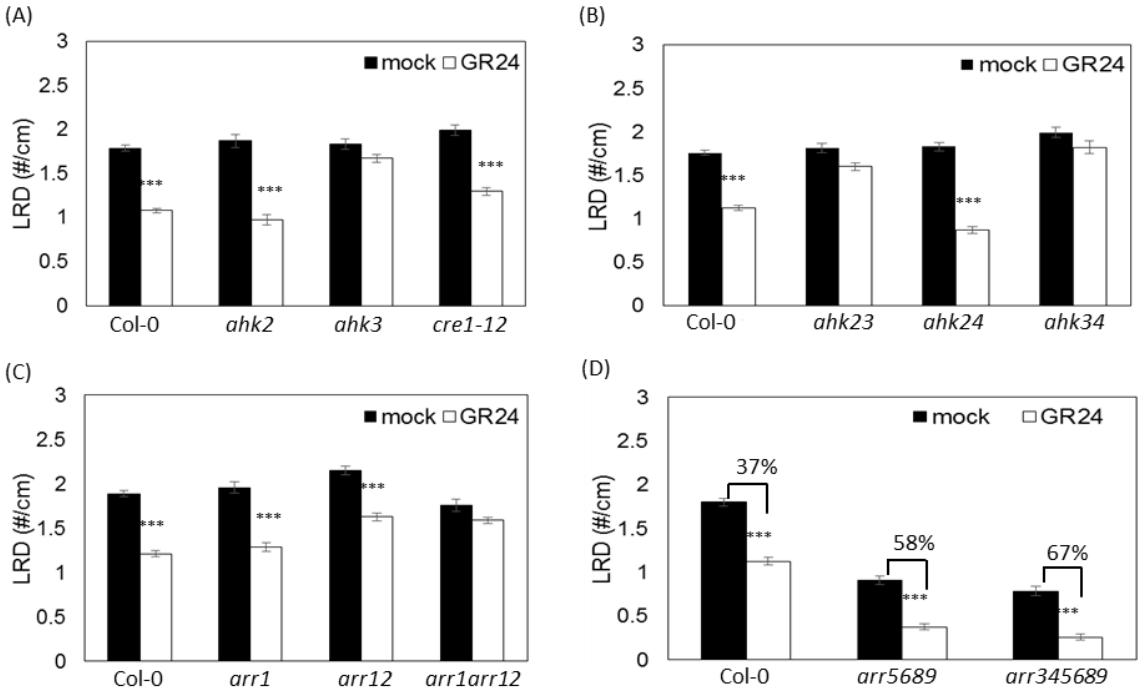

Figure 2. Effects of GR24 on cytokinin perception and signaling mutants.

LRD of single cytokinin receptor mutants (A), double cytokinin receptor mutants (B), B-type response regulators *ARR1*, *ARR12* and *ARR1ARR12* (C), and mutants in higher-order A-type response regulators (D) upon GR24 treatment. Data presented are means ± SE of three biological repeats ($n > 20$). ***$P < 0.001$, according to ANOVA mixed-model statistical analyses.

The modified sensitivity to GR24 of *ahk3*, *arr1arr12*, and *shy2* mutants is due to changes in the auxin landscape

The AHK3-ARR1ARR12 cytokinin signaling pathway has been shown to act upstream of *SHORT HYPOCOTYL2* (*SHY2*) to control root differentiation (Dello Ioio *et al*., 2007, 2008) and, additionally, the *shy2* loss-of-function mutant to be insensitive to GR24 as well (Koren *et al*., 2013). To elucidate why mutants in the *AHK3-ARR1ARR12-SHY2* module are affected in their

GR24 sensitivity, the GR24 phenotype was examined in different *pin* mutants, because *SHY2* has been described to specifically repress *PIN1, PIN3, PIN5,* and *PIN7*, whereas cytokinin treatment downregulated *PIN1* and *PIN3,* but upregulated *PIN7* expression (Dello Ioio *et al*., 2007; Růžička *et al*., 2009). First, the GR24 effect on LRD of mutations in *PIN1*, *PIN3*, *PIN5,* or *PIN7* was analyzed. The decrease in LRD of the *pin7* mutants was only minor upon GR24 treatment, indicating that mutation in *PIN7* reduced the root sensitivity to GR24 (Fig. 3A), but the LRD reduction of the *pin1-613* mutants was significantly higher than that in WT plants (Fig. 3B). For the *pin3-3* and *pin5-3* mutants, the LRD did not differ from that of WT plants (Fig. 3C).

Hence, these results demonstrate for the first time that interference with the polar auxin transport through modification of *PIN1* or *PIN7* expression modulates the LR response to exogenous GR24. Moreover, these data are in line with the observation of Růžička *et al*., 2009 that CK induced *PIN7* expression and reduced *PIN1* expression.

Previously, it has been demonstrated that prolonged treatments of GR24, but not short treatments, influenced the expression of *PIN1*, *PIN3* and *PIN7* in the root meristem, however the expression in the whole root has not been assesed before (Ruyter-Spira *et al*., 2011; Shinohara *et al*., 2013). Therefore, the effect of GR24 on the transcription of *PIN1* at the hypocotyl-root junction, where LR emergence is mostly affected by GR24 treatment, was investigated. After 7 days of growth of *pPIN1:GUS* seedlings, the impact of GR24 on the *PIN1* expression was analyzed by GUS staining. Interestingly, the *PIN1* expression was affected in a spatiotemporal way (Fig. 3D,E): in the mature root zone, i.e. the closest to the shoot, the expression in the vasculature was lower upon GR24 treatment than that under mock conditions (Fig. 3D). In developing LRs in the upper root part, the *PIN1* expression was also slightly lower than that of mock-grown roots, in contrast

to developing LRs at younger stages, i.e. near the root meristem (Fig. 3E). For p*PIN7:GUS* seedlings, no clear changes in the expression patterns were observed (data not shown).

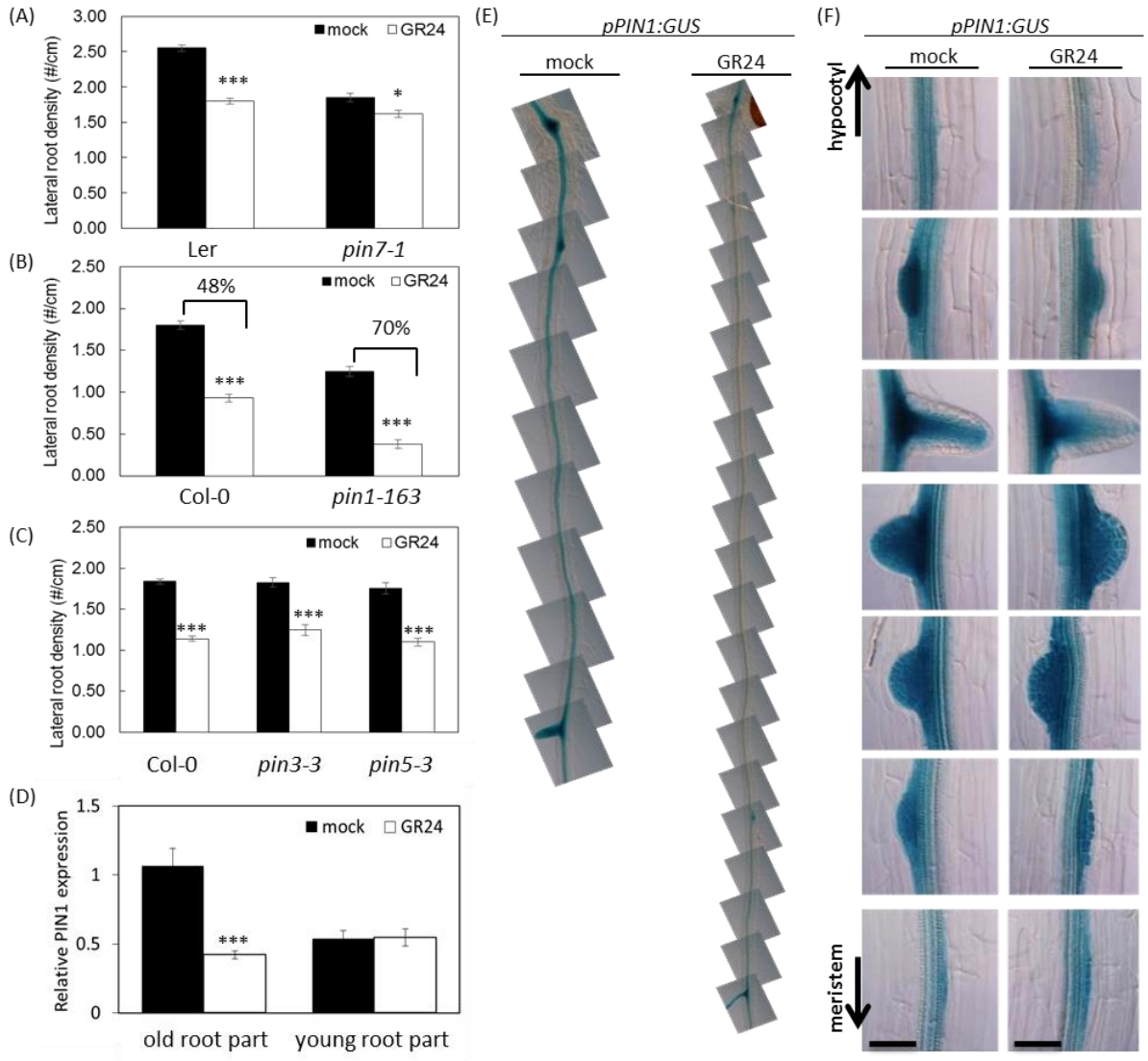

Figure 3. Interrelation between the polar auxin transport and the GR24 effect on LR development.

(A-C) LRD of *pin7-1*, *pin1-613*, *pin3-3,* and *pin5-3* mutants compared to WT grown in the presence or absence of GR24. Data presented are means ± SE of three biological repeats (n > 20). (D) p*PIN1:GUS* expression patterns of plants grown with and without GR24 application, 7 days after growth. Frames until the first emerged LR are shown. (E) Expression of *PIN1* with p*PIN1:GUS* plants during different stages of LR development under mock and GR24 treatment. ***P < 0.001, *P < 0.05, according to ANOVA mixed-model statistical analyses. Scale bars = 40 µm.

Thus far, the data demonstrate that mutations in the *AHK3-ARR1ARR12* cytokinin signaling module and in the auxin transport genes (*PIN1* and *PIN7*) affect the root sensitivity to GR24 and that GR24 influences auxin homeostasis by downregulating the expression of auxin transporters, in agreement with the reported decreased PIN protein levels in the root upon prolonged treatments with high concentrations of GR24 (Ruyter-Spira *et al*., 2011).

To further investigate how the auxin environment alters the GR24 effect, the GR24 response was examined in plants overexpressing *YUCCA* with concomitantly increased free auxin levels (Zhao *et al*., 2001). The LRD of *YUCCA1-D* plants did not decrease upon GR24 treatment, indicating that enhanced endogenous auxin levels also modulate the GR24 response in roots (Fig. 4A). Also 35S:PIN1-overexpressing plants that have highly increased frequencies of root primordia with retarded growth were analyzed (Benková et al., 2003). The typical GR24-mediated decrease in LRD was no longer visible, but rather an increase in LRD was observed (Fig. 4B). Moreover, when the foliar auxin source that determines the outgrowth potential of LRs (Bhalerao *et al*., 2002; Ljung *et al*., 2005) was removed by decapitation after 6 days of growth and when these plants were subsequently treated with GR24 for 5 days, the effects disappeared on both the *PIN1*-overpressing lines (increase in LRD) and the WT (decrease in LRD), indicating that shoot-derived auxin is important for the GR24 responses in roots (Fig. 4D). Application of IAA in these experiments (see Materials and Methods) revealed that shoot-derived auxin mediated the effect, because it complemented the phenotype of decapitated plants (Fig. 4D). Altogether, the functional data demonstrate that shoot-derived auxin controls the effect of GR24 on lateral rooting in *Arabidopsis*, as previously hypothesized based on the analysis of *PIN1* expression analyses (Ruyter-Spira *et al*., 2011).

All mutants with GR24-insensitive root responses, i.e. *ahk3, arr1arr12,* and *shy2-24*, display an enhanced *PIN1* expression (Dello Ioio *et al*., 2007, 2008; Zhang *et al*., 2011) that might cause their insensitivity. This hypothesis was tested by applying low concentrations (100 nM) of NPA, a polar auxin transport inhibitor (Himanen *et al*., 2002) and the LRD response was analyzed under mock and GR24 treatment after 9 days of growth (Fig. 4E). Addition of this low concentration of NPA had no impact on the LRD (Fig. 4E). However, when the *ahk3* and *shy2-24* mutants were grown on plates supplemented with NPA as well as GR24, the LRD was lower than that of roots grown under mock conditions or supplemented with GR24 or NPA alone, implying that treatment with NPA rendered the mutant plants responsive to GR24 again. For Col-0, no additional effect was seen when the roots were treated with both NPA and GR24.

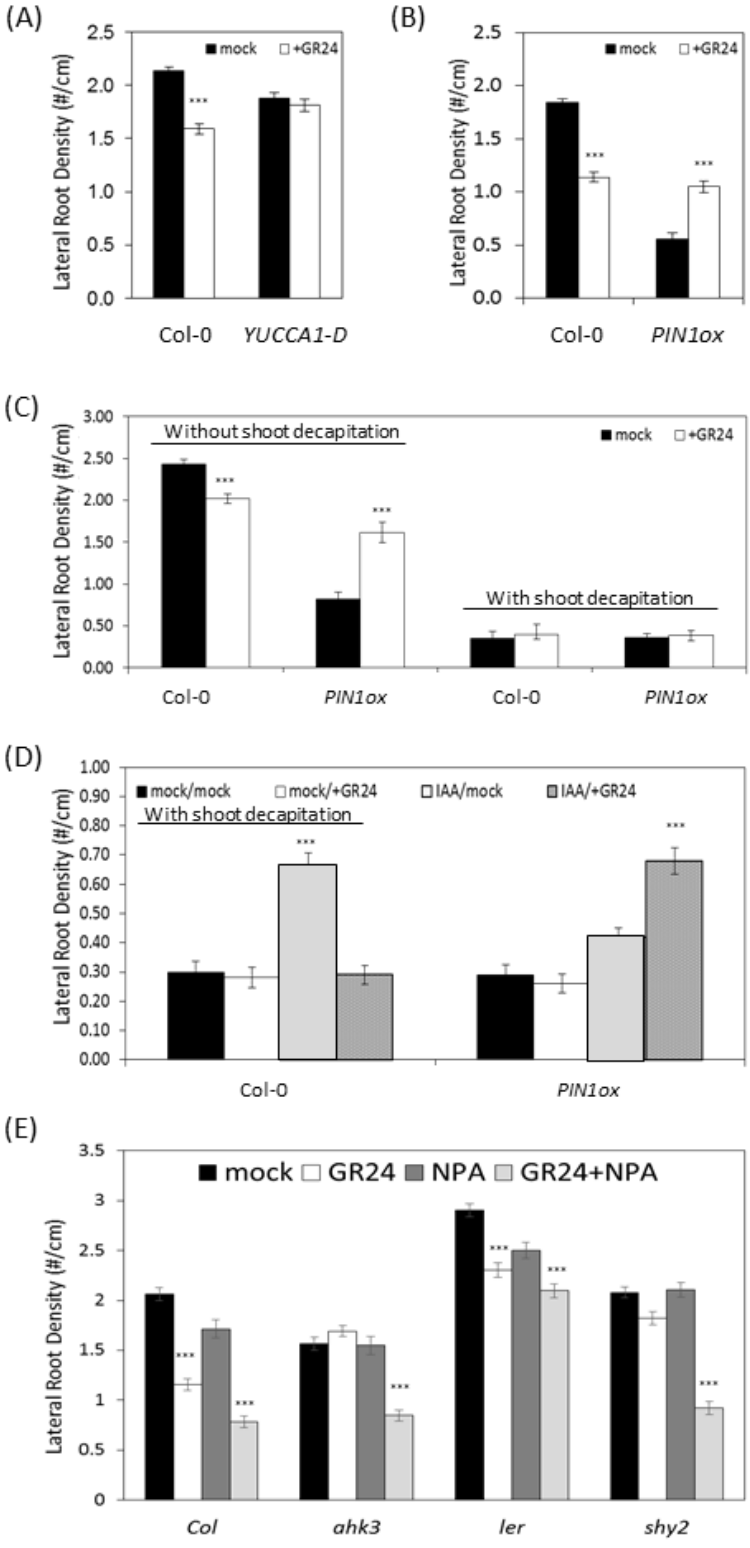

Figure 4. Dependence of GR24 action on the plant auxin status.

(A) LRD of WT and *YUCCA*-overexpressing (*YUCCA1-D*) plants, grown with and without GR24. (B) LRD of WT and *PIN1*-overexpressing (*PIN1ox*) plants, grown with and without GR24. (C) LRD of Col-0 and *PIN1ox*

plants with and without shoot decapitation, grown in the presence or absence of GR24. (D) LRD of Col-0 and *PIN1ox* plants with decapitation and with and without apically applied IAA grown in the presence or absence of GR24. Mock/mock: decapitated plants grown in absence of GR24 and without applied IAA; mock/+GR24: decapitated plants grown in presence of GR24 and without applied IAA; IAA/mock: decapitated plants grown in absence of GR24 and with apically applied IAA; IAA/+GR24: decapitated plants grown in presence of GR24 and with apically applied IAA. (E) LRD of Col-0, *ahk3,* L*er* and *shy2-24* mutants upon treatment with mock, GR24, NPA, or NPA+GR24. Data presented are means ± SE of three biological repeats ($n > 20$). ***$P < 0.001$, according to ANOVA mixed-model statistical analyses.

DISCUSSION

Several aspects of the root system architecture are modulated by SLs (for reviews, see Cheng *et al*., 2013; Rasmussen *et al*., 2013; Koltai, 2014). Here, GR24 was found to control LR development spatiotemporally and to interplay with cytokinin that, just like SLs, regulates LR development.

The method established to build a developmental map of all possible initiated LRs combines the *GATA23* marker gene for initiation of prebranching sites, i.e. pericycle-derived LR founder cells that are predestined to start cell division for LR development, and LR positioning (Malamy and Benfey, 1997; De Rybel *et al*., 2010). Together with the determination of the position of each event along the main root, a precise developmental map was made providing location and developmental stage of each LR event, thereby revealing that the main effect of GR24 on the development of LRs concerned their emergence. This observation concurs with previously published work, although the proposed specific blockage at stage V of LR development was not detected (Ruyter-Spira *et al*., 2011).

On the 9-DAG map, the LRs were mainly, but not exclusively, situated close to the root–shoot junction that no longer emerged under GR24 treatment. Accordingly, the distance between the hypocotyl-shoot junction and the first emerged LRs was longer in GR24-grown roots than that of control roots. This MAX2-dependent effect is in accordance with its essential function in SL signaling. Hence, GR24 might affect specifically the emergence of the LRs that develop first and are positioned in the older root parts. This spatiotemporal effect was also seen on the *PIN1* expression pattern in the root. Although the reason for this effect needs to be investigated in the future, the disappearance of the SL receptor might be the underlying cause, because GR24 treatment reduces the D14 protein abundance in roots (Chevalier *et al*., 2014).

Additionally, a small, but significant, decrease in prebranch sites was visible, whereas GR24 had no appreciable effect on LR initiation. Hence, the previously detected GR24 effect on LR initiation (Kapulnik *et al*., 2011b; Ruyter-Spira *et al*., 2011) is possibly due to an impact on prebranching. Prebranch sites are established by a periodic oscillation of auxin concentrations accompanied by fluctuation in specific gene expression (De Smet *et al*., 2007; Moreno-Risueno *et al*., 2010). This oscillating pattern has been found to be mediated by a carotenoid compound, distinct from SLs (Van Norman *et al*., 2014). In agreement with our data, this study also showed that the *max2* mutants exhibit an increased LR capacity (Van Norman *et al*., 2014). It would be interesting to analyze whether GR24, as a mimic of SLs or related compounds, modulates the periodic oscillation of auxin to cause the small effect on prebranching. Furthermore, independently of SLs, fewer LR events are observed on the same position of main root part at 9 DAG than at 4 DAG, possibly indicating that not all primed sites develop into LRs. In-depth experiments are currently being done to understand how this variable might control the influence of the environment on LR development.

Cytokinins have been identified as endogenous repressors of LR development in a close interplay with auxin (Benková *et al*., 2003; Li *et al*., 2006; Laplaze *et al*., 2007). Here, the GR24 effect on LR development has been shown to require the functional cytokinin receptor AHK3, but not AHK2 and AHK4/CRE1. The dependence on *AHK3* and not on *AHK4* is remarkable, because *AHK4* has been implicated in LR patterning along the main root (Marhavý *et al*., 2011), whereas *AHK3* and the two immediately downstream B-type response regulator genes, *ARR1* and *ARR12*, play an important role in determining the root meristem size (Dello Ioio *et al*., 2007, 2008). Also in the experimental setup, the double mutant *arr1arr12* had no LR response toward GR24, implying that the same cytokinin module (*AHK3-ARR1ARR12*) that rules root meristem differentiation also governs the GR24 action on LR development. *AHK3* is involved in meristem differentiation by transcriptional control of the auxin-induced *SHY2/IAA3* gene (Dello Ioio *et al*., 2007, 2008). The typical reduction in lateral rooting upon GR24 treatment was indeed not seen in the *shy2-24* loss-of-function mutants (Koren *et al*., 2013), supporting the hypothesis that the *AHK3-ARR1ARR12* module acts through *SHY2* to induce GR24 insensitivity.

The *AHK3-ARR1ARR12-SHY2* module negatively influences *PIN1/PIN3/PIN5/PIN7* expression (Dello Ioio *et al*., 2007, 2008), whereas cytokinin treatment downregulates *PIN1/PIN3/PIN5*, but upregulates *PIN7* expression (Laplaze *et al*., 2007; Růžička *et al*., 2009). These changes in *PIN* gene expression and their consequences on the polar auxin transport might be the underlying cause for the GR24 insensitivity of the mutants. Several *PIN* mutants had a modified sensitivity to GR24: *pin3* and *pin5* mutants still displayed a reduced LR development upon GR24 treatment, whereas *pin7* mutants were only slightly responsive to GR24 and *pin1-613* mutants were hypersensitive in agreement with the opposite influence of cytokinins on their expression. In addition, treatment of *ahk3* and *shy2-24* with NPA made them sensitive again to GR24. Hence,

the changes in *PIN* gene expression, such as the *PIN1* overexpression observed in these mutants (Dello Ioio *et al*., 2007, 2008; Zhang *et al*., 2011) with an enhanced polar auxin transport as a result, might be the reason that GR24 does not reduce the LRD in these mutants.

Moreover, the data support the central role of auxin transport for the SL action. Based on the exogenous auxin addition and phosphate level modulation, the auxin content in roots has been shown to determine its responsiveness toward GR24 (Ruyter-Spira *et al*., 2011). Indeed, endogenous overproduction of auxin via overexpression of *YUCCA* could make the LRD unresponsive to GR24. Interestingly, *PIN1*-overexpressing plants no longer displayed a reduced LRD when treated with GR24, but an opposite phenotype with an increased LRD was observed. Also in the shoot, depending on the auxin transport landscape, GR24 could have positive or negative effects on shoot lateral branching by depleting PIN1 from the membranes of xylem parenchyma cells of inflorescence stems (Shinohara *et al*., 2013). In addition, it has been shown that GR24 has a different effect on LR development depending on the growth conditions: it inhibits LR development under conditions with sufficient Pi, while it induces it under low Pi condition (Ruyter-Spira *et al*., 2011). Hence, overexpression of *PIN1* causes a similar effect on GR24 responses as phosphate-limiting conditions does: an increase, rather than a decrease in LRD.

In conclusion, the data presented imply that the GR24 regulates LR development in a spatiotemporal manner with the strongest effect on emergence of the first developed LR positioned close to the root-shoot junction. This effect is tightly integrated into the auxin-cytokinin network that rules the root architecture with the polar auxin transport capacity as a central player on which both cytokinin and GR24 act.

MATERIALS AND METHODS

Plant material and growth conditions

The *pin7-1* mutant from *Arabidopsis thaliana* (L.) Heyhn. is in Landsberg *erecta* (L*er*) background, whereas the other lines described are in Columbia-0 (Col-0) background. The plant material used has been described previously: *ahk2-2, cre1-12,* and *ahk3-3* (Higuchi *et al*., 2004); *ahk2ahk3, ahk2ahk4*, and *ahk3ahk4* (Riefler *et al*., 2006); *arr1, arr12* and *arr1arr12* (Mason *et al*., 2005); *arr3arr4arr5arr6,* and *arr3arr4arr5arr6arr8arr9* (To *et al*., 2004); *pin1-613* (Bennett *et al*., 2006); *35S:PIN1-GFP* (Růžička *et al*., 2007); *pin3-3* (Friml *et al*., 2002); *pin5-3* (Mravec *et al*., 2009); *pin7-1* (Friml *et al*., 2003); *shy2-24* (Tian and Reed, 1999); *proAHK3:GUS* (Higuchi *et al*., 2004); *proPIN1:GUS*, *proPIN7:GUS*, and; *proPIN7:PIN7-GFP* (Blilou *et al*., 2005); *pGATA23:NLS-GFP-GUS* (De Rybel *et al*., 2010); and *YUCCA* (Zhao *et al*., 2001).

Seeds were surface-sterilized for 5 min in 70% (v/v) ethanol, 0.05% (v/v) sodium dodecyl sulfate (SDS) solution, then incubated in 95% (v/v) ethanol for 5 min, and plated on half-strength Murashige and Skoog (½MS) medium (1% [w/v] sucrose and 0.8% [w/v] agar). Plants were stratified at 4°C for 2 days, transferred to a growth chamber at 21°C (16-h light/8-h dark photoperiod). A racemic mixture of GR24 was supplemented to the growth medium at the start of the experiment and plants were grown for the indicated time. All the experiments were repeated three times. Chemical compounds were added in the following concentrations, except indicated otherwise: 1 μM GR24 and 0.1 μM 1-*N*-naphthylphthalamic acid (NPA).

Phenotypic analysis and statistics

After 9 days of growth, LRs were counted under a binocular microscope (Leica S4E; http://www.leica-microsystems.com) and root length was measured with ImageJ (http://rsb.info.nih.gov/ij). Both values were used to calculate the LRD. For the decapitation experiments, seedlings were grown for 6 days, whereafter the shoot was removed as described (Forsyth and Van Staden, 1981). The bottom part was transferred to ½MS medium with or without 1 µM GR24. For the complementation with indole-3-acetic acid (IAA), agar blocks (0.5 cm^3) containing solidified growth medium with and without 10 µM IAA were added to the decapitated site and the LRD was analyzed 5 days later. Replicate means were subjected to statistics by analysis of variance (ANOVA; SAS Institute Inc., Cary, North Carolina, USA; http://www.sas.com).

Stage determination by GATA23 expression analysis

pGATA23:NLS-GFP-GUS seeds were put on medium supplemented with 1 µM GR24 or with the same volume of acetone as control and were stratified for 2 days at 4°C. Seedlings were grown vertically under continuous white light at 21°C. At 4 days after germination (DAG), half the seedlings were harvested for analysis, whereas for the remaining seedlings, the position of the main root tip was marked and the plates were transferred back to the growth room. At 9 DAG, the root parts above the mark were harvested. Samples were stained with β-glucuronidase (GUS), cleared as described (Malamy and Benfey, 1997) and analyzed under the microscope (see below). For the calculations of the percentage of initiated sites, the total average of initiations at 9 DAG was divided by the total average sites present at 4 DAG. Likewise for the calculations of the percentage of emerged sites, the total average of emerged LRs at 9 DAG was divided by the total average sites present at 4 DAG.

Histochemical analysis of GUS activity

Whole seedlings were stained in multiwell plates as described (Jefferson *et al*., 1987). Samples were cleared as described (Malamy and Benfey, 1997) and were analyzed by a differential interference contrast microscope (Olympus BX51; http://www.olympusmicro.com). Alternatively, samples were mounted directly in chloral hydrate solution (chloral hydrate:water:glycerol, 8:3:1) and microscopically analyzed.

Accession numbers

The Arabidopsis Genome Initiative locus identifiers for the genes characterized in this study are: *AHK3* (AT1G27320), *SHY2* (AT1G04240), *PIN1* (AT1G73590), *PIN7* (AT1G23080), and *YUCCA1* (AT4G32540). Germplasm identification numbers for the seeds are: *ahk2* (*ahk2-2tk*), *ahk3-3* (SALK_069269), *cre1-12* (SALK_048970), *ahk2ahk3* (*ahk2-5ahk3-7*), *ahk2ahk4* (*ahk2-5cre1-12*), *ahk3ahk4* (*ahk3-7cre1-2*), *arr1-2* (N6368), *arr12-1 (CS6978)*, *arr1arr12* (*arr1-3;arr12-1*), *pin1-613* (SALK_047613), and *pin5-3* (SALK_021738).

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SUPPLEMENTARY DATA

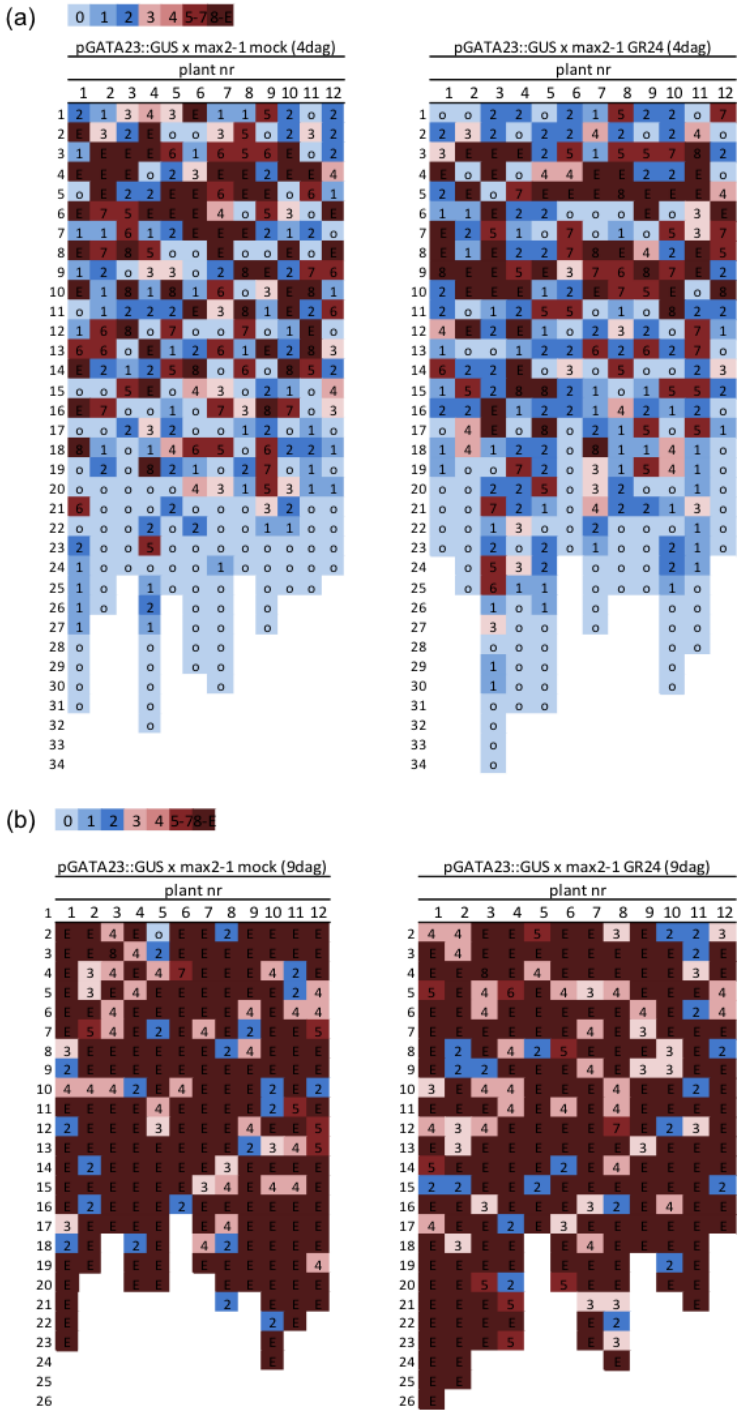

Figure S1. Stages of LR primordia via *GATA23-GUS* staining in *max2-1* under mock and GR24 treatment at 4 (a) and 9 DAG (b). All events, possibly leading to emerged lateral roots, were scored in individual plants, color-coded, and for each plant, vertically ordered from the closest to the hypocotyl (up) downward to the meristem (down). Data of one representative experiment are shown. The experiments were repeated three times with similar results.

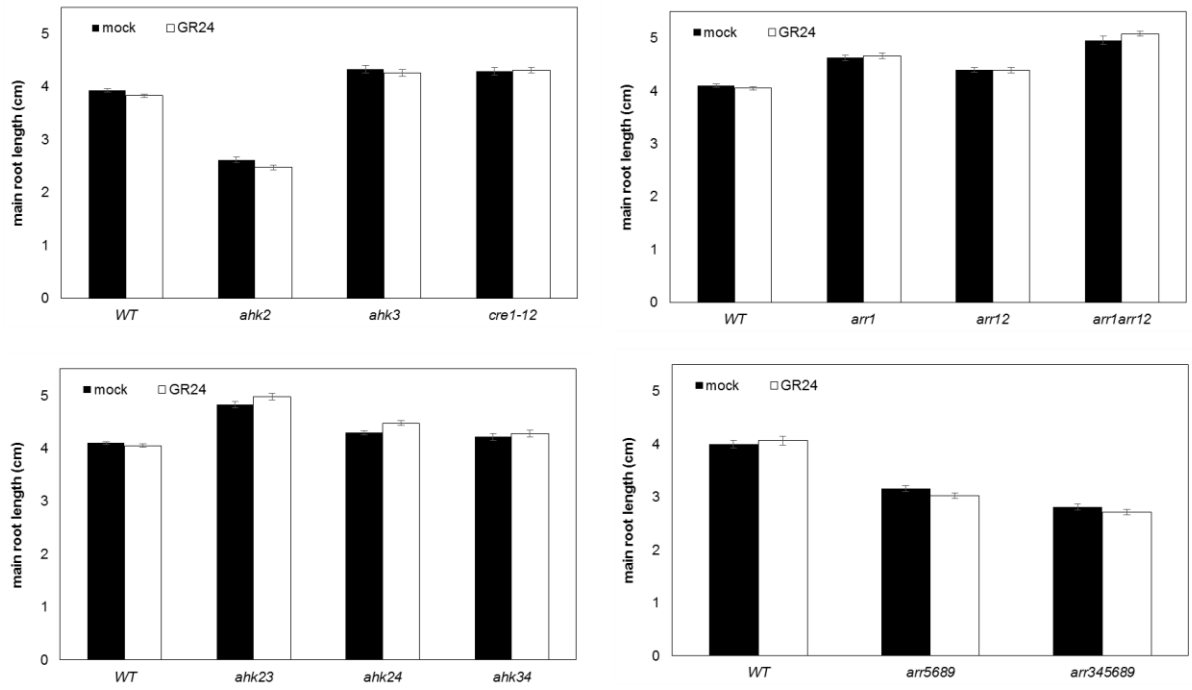

Figure S2. . Main root lengths of WT and cytokinin receptor and signal transduction mutants under mock and GR24 treatment. Data presented are means ± standard errors of three biological repeats (n > 20). **P*-value <0.05, according to ANOVA mixed-model statistical analyses.

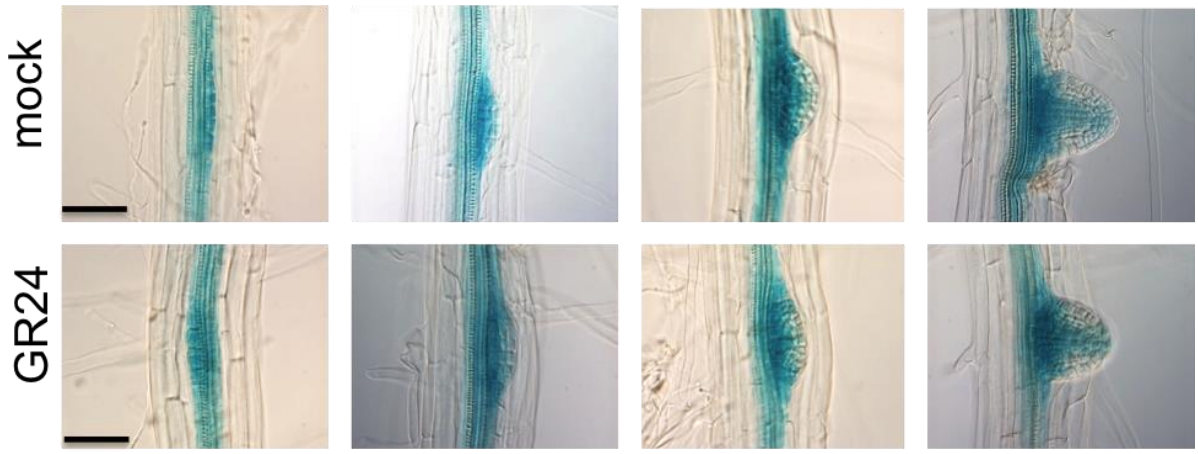

Figure S3. *pAHK3-GUS* expression patterns in LR primordia at different developmental stages under mock and GR24 treatment.

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Chapter III

Crosstalk between strigolactones and jasmonate and ethylene during lateral root development

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ABSTRACT

The plant root system is important for water and nutrient uptake and anchoring of plants in the soil. Lateral roots (LRs) are an important feature in the root system architecture in which phytohormones, generally interacting with each other, play pivotal roles. The most recently described plant hormones, strigolactones (SLs), have been demonstrated to repress LR development under sufficient nutrition conditions, whereas LR development is promoted and primary root growth inhibited by the hormone jasmonate (JA). However, the mechanism by which SLs interact with other hormones to regulate LR development remains unclear. In the present study, by using a spatiotemporal analysis, we show that JA inhibits LR outgrowth and triggers LR priming. SLs proposed to be involved in the JA impact on LR in a MORE AXILLARY GROWTH2 (MAX2)-dependent manner. Transcriptional and translational analyses showed that JA up-regulates the SL signaling genes. Hence, we propose that the effect of JA on root priming might be executed via a JA-dependent induction of SL signaling genes. In contrast, JA does not seem to be involved in the SL effect on LR development. Ethylene is another important plant hormone that been reported to interact with SL to regulate root hair elongation. Here, we investigated the interaction between ethylene and SLs in LR development. The experiments revealed that the lack of direct interplay between these two hormones in the LR development process, because the ethylene signaling mutants respond to SLs and the SL biosynthesis and signaling mutants respond to ethylene.

INTRODUCTION

The root system of higher plants consists generally of a primary root from which lateral roots (LRs), adventitious roots and root hairs emerge. The growth of the root system is highly plastic and depends on various environmental and endogenous factors that allow plants to compete for resources in the soil and to adapt to constantly changing growth conditions (Hodge et al., 2009; Jarzyniak and Jasinski, 2014; Grienenberger and Fletcher, 2014). LR development is a critical determinant for plant survival because of the potential to increase branching and enhance the exploratory capacity of roots to seek nutrients. LR development has been intensively studied in *Arabidopsis thaliana*: they originate from xylem pole pericycle cells (Malamy and Benfey, 1997; Dubrovsky et al., 2008), whereafter the LR primordia (LRP) emerge through the endodermis, cortex, and epidermis of the primary root to form a new organ (De Smet et al., 2012). The process of LR development is strictly regulated by endogenous and exogenous cues, among which the phytohormones play pivotal roles.

The central hormone for root development is auxin and various auxin signaling pathways have been shown to be involved at various steps during LR development (Lavenus et al., 2013). However, other hormones play a role as well, often through impinging on the auxin-dependent regulatory processes, as is the case for jasmonates (JAs). Jas that consist of a group of lipid-derived compounds from which the isoleucine derivate has been described as the biological active one (Staswick and Tiryaki, 2004; Fonseca et al., 2009) were discovered as crucial components of the plant defense signaling system against insects and pathogens (Kessler et al., 2004; Browse and Howe, 2008). However, based on mutant analysis and impact of exogenous JA, JAs play also various roles in plant and in root development. In *Arabidopsis*, exogenous JA treatment inhibits primary root growth, probably due to an arrest in mitosis (Staswick et al., 1992; Feys et al., 1994). The JA perception mutant *coronatine insensitive1* (*coi1*) relieved the JA-induced inhibition of root growth (Xie et al., 1998), whereas JA signaling mutants, such as

jasmonate-insensitive1 (*jin1)/myc2* and *jasmonate-insensitive3* (*jai3*), largely reduced root growth inhibition due to JA application (Lorenzo et al., 2004). Additionally, JAs also enhance LR formation by inducing the expression of the auxin biosynthesis gene *ANTHRANILATE SYNTHASE ALPHA SUBUNIT1* (*ASA1)* and by affecting local auxin accumulation in the basal meristem via modulation of the polar auxin transport (Sun et al., 2009, 2011). In addition, JA and auxin have been reported to share common signaling pathway components (Tiryaki et al., 2002; Ren et al., 2005; Pauwels et al., 2010), interplaying through the action of *AUXIN RESPONSE FACTORs* (*ARFs)* (Nagpal et al., 2005). In contrast, the JA signaling pathway also affects auxin homeostasis via adjustment of the expression of *YUCCA8* (*YUC8*) and *YUC9* (Hentrich et al., 2013). In the regulation of root hair (RH) development, RH formation has been reported to be stimulated by JAs, possibly through synergistical action with ethylene (Zhu et al., 2005).

Ethylene, a simple gaseous phytohormone, is also involved in the regulation of root development (Negi et al., 2008; Ivanchenko et al., 2008). In *Arabidopsis*, ethylene is sensed by a family of endoplasmic reticulum (ER)-localized receptors that act as negative regulators of the signaling pathway (Chang et al., 1993, Hua et al., 1995, Hua and Meyerowitz, 1998, Hua et al., 1998 and Sakai et al., 1998; reviewed by Merchante et al., 2013). Downstream of the receptors, a Raf-like Ser/Thr protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), also functioning as a negative regulator of the pathway, controls downstream processes (Kieber et al., 1993) through ETHYLENE INSENSITIVE2 (EIN2), a membrane protein that is a central transducer of the ethylene signaling cascade (Alonso et al., 1999). Further downstream, EIN3 and EIN3-LIKE1 (EIL1) sense the ethylene signal from EIN2 that operates as master transcription factor (TF) to regulate the expression of targets genes, such as *ETHYLENE RESPONSE FACTOR1* (*ERF1*), that subsequently regulate hundreds of ethylene-responsive genes (Chao et al., 1997; Solano et al., 1998). Ethylene has been demonstrated to inhibit LR formation, because mutants in the

ethylene signaling pathway, such as *ctr1*, or mutants overproducing ethylene, such as *ethylene overproducer1* (*eto1)*, have a reduced capacity to form LRs (Negi et al., 2008; Strader et la., 2010). Moreover, treatment with ethylene or 1-aminocyclopropane-1-carboxylic acid (ACC) reduced LR initiation in both *Arabidopsis* and tomato (*Solanum lycopersicum*) (Negi et al., 2008; Ivanchenko et al., 2008; Strader et al., 2010). By contrast, the ethylene-insensitive *ein2* and *ein3* mutants and the ethylene receptor mutant *etr1* that confers dominant ethylene insensitivity, form more LRs (Negi et al., 2008; Negi et al., 2010). The ethylene impact on LR formation has been demonstrated to act through the modulation of auxin transport and accumulation patterns (Negi et al., 2008; Lewis et al., 2011; Muday et al., 2012). In addition, based on the responses to the ethylene precursor ACC and to the biosynthesis inhibitor components as well as through phenotypical analysis of ethylene mutants, such as *ctr1* and *eto*, ethylene has been suggested to stimulate RH formation (Dolan et al., 1994; Tanimoto et al., 1995; Pitts et al., 1998; Cao et al., 1999). Besides LR development and RH formation, root growth has also been found to be influenced by ethylene, during which an interplay with auxin resulted in inhibitory effects on root growth (Swarup et al., 2002; Růžička et al., 2007; Strader et al., 2010).

Strigolactones (SLs) are the most recently discovered plant hormones and modulate RH growth, primary root growth as well as LR development (Ruyter-Spira et al., 2011; Kapulnik et al., 2011a, Kapulnik et al., 2011b; Waldie et al., 2014). Whether the developmental pathways are induced or repressed by SLs depends on the nutrient conditions and the auxin status of the plant (Ruyter-Spira et al., 2011; Kapulnik et al., 2011a; Arite et al., 2012; Guan et al., 2012; Kretzschmar et al., 2012). Application of the synthetic strigolactone GR24 increased the number of cells in the root meristem, resulting in a promotion of primary root growth in a manner dependent on the SL signaling component MORE AXILLARY GROWTH2 (MAX2) (Ruyter-Spira et al., 2011). This primary root growth promotion depended on the growth conditions and

on the applied GR24 concentration (Ruyter-Spira et al., 2011; Shinohara et al., 2013). Under nutrient-rich conditions, exogenous GR24 caused a decrease in LR development also in a *MAX2*-dependent manner (Kapulnik et al., 2011a; Ruyter-Spira et al., 2011). Further analysis has shown that the inhibitory effect was caused by a reduction in LR initiation as well as in LR outgrowth (Kapulnik et al., 2011a; Ruyter-Spira et al., 2011). Part of the SL action mechanism on the main root growth and LR development might be due to changes in the auxin transport capacity via a reduced expression of the *PIN-FORMED1* (*PIN1*) expression (Ruyter-Spira et al. 2011). The influence of SLs on RH development has also been investigated. In *Arabidopsis* roots, SLs have been suggested to stimulate RH elongation in a MAX2-dependent, but also ethylene-dependent, manner (Kapulnik et al., 2011b), whereas SLs and auxin were shown to act independently to control RH growth (Kapulnik et al., 2011b).

Plant hormones operate in a network of interacting responses rather than through isolated linear pathways. Whereas auxin is a key hormone for LR development, other hormones are involved as well by acting as positive or negative regulators. Although the interaction between JAs, ethylene, and auxin that influence LR development has been well studied, the interplay of JAs and ethylene with SLs to control LR development has not been assessed yet. Here we show that ethylene and SLs act independently of each other to control root growth and LR development, whereas there is an interplay between JA and SLs.

RESULTS

The effect of GR24 on root length and lateral root density of mutants and transgenic lines affected in JA biosynthesis or signaling.

To investigate whether JAs and SLs interplay to control LR development, the impact of addition of the synthetic SL analog, GR24, on root length and LR number was analyzed in mutants and transgenic lines affected in JA biosynthesis or signaling. The mutants used were *coronatine insenstitive1-16* (*coi1-16*) affected in the JA receptor (Ellis and Turner, 2002), *jai3-1* that is a dominant mutant resulting in the formation of a truncated JASMONATE-ZIM-DOMAIN3 (JAZ3) protein, no longer subjected to COI1-dependent degradation (Chini et al., 2007), *ninja* affected in the negative regulator *Novel Interactor of JAZ* (*NINJA*) (Pauwels et al., 2010; Acosta et al., 2013), *jin1-2* affected in the TF *MYC2* that is part of the core signaling module of the JA signaling pathway (Lorenzo et al, 2004), and the triple mutant *myc2myc3myc4* (Fernandez-Calvo et al., 2011), as well as of the *35S:MYC2* transgenic line (Lorenzo et al., 2004) and the *JAR1-OX* overexpressing line (Gutierrez et al., 2012) that result in elevated MYC2-dependent signaling and incresed JA-Ile production, respectively.

Seeds of these lines together with the wild-type (WT) Columbia-0 (Col-0) *Arabidopsis* strain were germinated on half-strength Murashige and Skoog (½MS) medium with and without 1 µM GR24 and grown for 9 days after 2 days incubation at 4°C. Subsequently, the LR number was counted and the main root length was measured with the ImageJ software (http://rsb.info.nih.gov/ij/), whereafter the LR density (LRD) was calculated by dividing the LR number by the main root length.

For the Col-0 WT plants, 1 µM GR24 did not significantly affect the primary root length as well as for all mutants tested. In addition, no significant difference between Col-0 and all mutants, except *coi1-16* and *ninja*, was observed in the primary root length when grown under mock conditions. The primary root length of *coi1-16* was longer than that of Col-0, wherease *ninja* was shorter (Fig. 1A).

Under mock conditions, the LRD of *coi1-16* and *myc2myc3myc4* was higher than that of Col-0 (*P* value <0.01 and <0.001, respectively), whereas it was lower in *35S:MYC2* and *JAR1-OX* (*P* values < 0.001). The LRD of the other mutants, such as *jai3*, *ninja*, and *jin1-2*, was similar to that of Col-0. When mock and treatment conditions were compared, the reduction caused by GR24 on all tested mutants, expect for *jin1-2* and *myc2my3myc4*, was around 20-40%, which was not significantly different to the reduction observed in the WT (Fig. 1B). In contrast, growth on GR24 caused a reduction in LRD of only 12% and 15% in the *jin1-2* mutant and *myc2myc3myc4* triple mutant, respectively, significantly lower than the 36% decrease observed in Col-0 (*P* value<0.05). Additionally, also the *35S:MYC2* transgenic plants behaved differently than the control plants. A decrease of 13% was observed that was also significantly smaller than for WT plants (*P* value<0.05).

These data indicate that the *MYC2* and related genes, but not the JA signaling pathway, might be involved in the regulation of the SL-mediated effect on LR development.

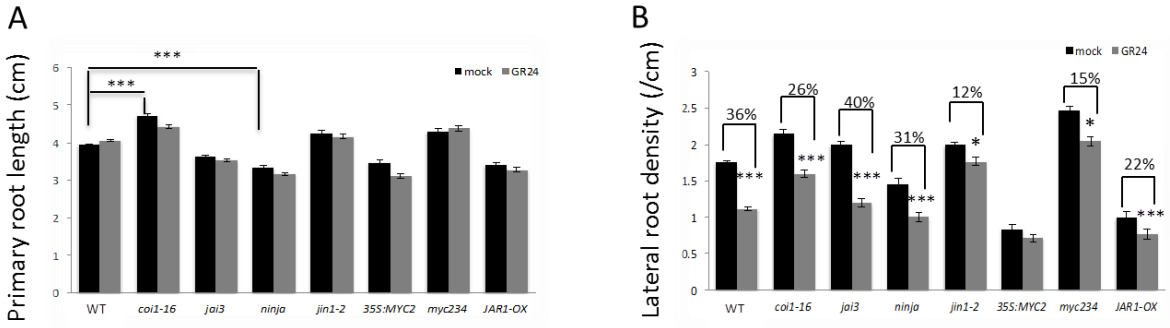


Figure 1. Effect of GR24 on mutants and transgenic lines affected in JA biosynthesis or signaling.

(A) Primary root length of Col-0 (WT) and various mutants and transgenic lines affected in JA biosynthesis or signaling grown in the presence or absence of GR24. **(B)** Lateral root density of the corresponding lines grown in the presence or absence of GR24. Comparisons were done between mock

and treatment. Represented data are means ± standard errors of three biological repeats (n > 20). *P value <0.05, ***P value<0.001, according to ANOVA mixed-model statistical analyses.

The effect of JA on the root length and lateral root density of strigolactone biosynthesis and signaling mutants.

To further examine a possible interplay between JAs and SLs in regulating root development, the effect of JA on the root system development was investigated in various mutants interrupted in SL biosynthesis and signaling. Here, *max1-1*, *max3-9*, and *max4-1* affected in the SL biosynthesis pathway (Stirnberg et al., 2002; Booker et al., 2004; Sorefan et al., 2003) and *dwarf 14* (*d14*) and *max2-1* affected in the SL signaling pathway were used (Nakamura et al., 2013; Stirnberg et al., 2002).

The inhibitory effect of exogenous JA on the root length has been well investigated (Staswick et al., 1992; Feys et al., 1994; Yan et al., 2007; Zhang and Turner, 2008). Indeed, 2 μM JA caused a decrease in the primary root length of approximately 50% (Fig. 2A) and this effect was observed in Col-0 as well as in the SL mutants (Fig. 2A), but addition of JA has been described to increase the LRD (Sun et al., 2009; Raya-Gonzalez et al., 2012). In agreement, 2 μM JA increased significantly the LRD of Col-0 for approximately 25%. Interestingly, this effect was not observed anymore in both the SL biosynthesis as well as signaling mutants (Fig. 2B). These findings suggest that SL biosynthesis and signaling might mediate the JA effect on LR development.

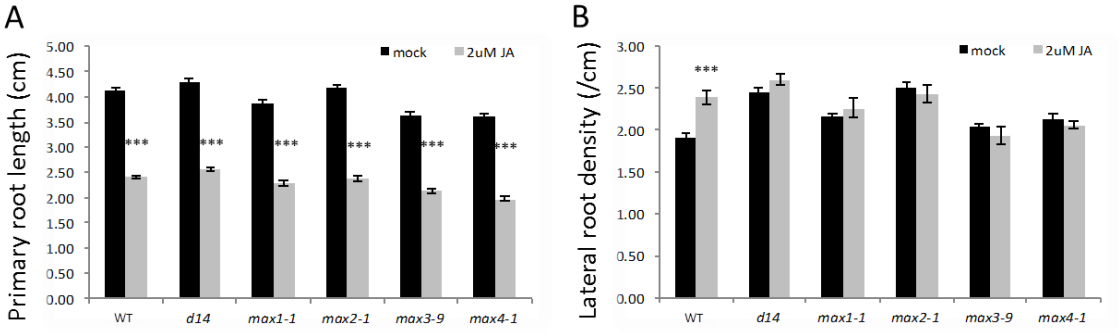


Figure 2. Effect of JA on mutants affected in SL biosynthesis or signaling.

(A) Primary root length of Col-0 (WT) and various mutants affected in SL biosynthesis or signaling grown in the presence or absence of JA. **(B)** Lateral root density of the corresponding lines grown in the presence or absence of JA. Seedlings were 9 days old. Comparisons were done between mock and treatment. Represented data are means ± standard errors of three biological repeats (n > 20). ***P value <0.001, according to ANOVA mixed-model statistical analyses.

Spatiotemporal insights into the effect of JA on lateral root development in Col-0 and *max2-1*

To further understand how SLs and JAs interplay to control LR development, we investigated the effect of JA on LR development in a spatiotemporal way. To this end, we established a method with the *pGATA23:NLS-GFP-GUS* as marker of LR founder cells (De Rybel et al., 2010) and according to the staging protocol of Malamy and Benfey (1997) (Fig. 3A, 3B). This method has been described in Chapter 2 and allows the visualization of all the stages from LR priming (stage 0) to emergence. Seeds of *pGATA23:NLS-GFP-GUS* were grown on ½MS medium with 1% (w/v) sucrose with or without 2 µM JA for 4 days after germination (DAG). Next, half of plants were harvested for GUS analysis and the position of the root tips was marked for the other part that continued to grow until 9 DAG. All LR events from plants at 4 DAG as well as 9 DAG were analyzed by GUS staining and staging. However, for the 9-DAG plants,

only the root part present at 4 DAG was used for the analysis. As a result, the comparison of 4-DAG and 9-DAG plants gave an insight into the temporal changes in LR development in the same area of the root.

For visualization, the data of each root were shown in a vertical way. A number was assigned to each LR site. Number 0 corresponded to a prebranch site that was stained by GUS, but that did not yet undergo cell division and numbers 1 to 8 corresponded to the previously described developmental stages (Malamy and Benfey, 1997). In addition, in the table, each site was color-coded: dark-brown corresponded to the VIII stage and emergence events, pink to stages V to VII, and bright to dark-blue to stage 0 until stage IV (Fig; 3A, 3B).

JA increased the LR number compared to that of plants grown under mock conditions. Indeed, as indicated (Fig. 3C and Supplemental Fig. 1), the total number of LR sites, including prebranching sites on JA-treated plants was higher than that of control plants at 4 DAG, but at 9 DAG anymore (Fig. 3C; Supplemental Fig. 2).

Next, for each developmental stage, the number of sites was counted for each plant grown on mock medium or in the presence of JA at 4 and 9 DAG. At 4 DAG, when the numbers of prebranching events of plants grown on JA were compared to mock treatment, JA-treated plants presented significantly more prebranching events (Fig. 3F), but not at 9 DAG (Fig. 3G). This effect was seen in both repeats. In contrast, the emerged patches were significantly reduced from 29% to 19% for mock and JA treatments, respectively (Fig. 3E). Correspondingly, when events at different stages were compared between mock and JA treatments, stage VIII and emergence events at 4 DAG were reduced (Fig. 3F) and this inhibitory effect could also be observed at 9

DAG (Fig. 3G). However, the total number of initiated patches of plants at 9 DAG was not affected between mock and JA treatments (Fig. 3D).

Hence, these data indicate that JA stimulates the number of LR events, but then inhibits its further development once the prebranching sites have been formed. These data provide us with much more in-depth insights and show that JA causes two opposite effects on LR development: induction of LR prebranch formation, but repression during outgrowth.

To understand how SLs might play a role in the JA effect on LR development, the *pGATA23:NLS-GFP:GUS* was crossed into the *max2-1* mutant (see Chapter 2) and the LRs were scored in the same manner as for Col-0. In contrast to the Col-0 background, the total LR sites, including prebranching sites, in the *max2-1* background were not affected by JA (Fig. 4A-4C), whereas the JA-dependent reduction in outgrowth was still present in *max2-1* (Fig. 4E). Correspondingly, when the number of sites at the different developmental stages was analyzed the promoting role of JA on prebranching events was not observed in the *max2-1* background at 4 DAG, but outgrowth was still inhibited both at 4 and 9 DAG (Fig. 4F and 4G, Supplemental Figure 2). These data indicate that SLs interfere with the JA impact on prebranching site formation early during LR development, but are not involved its effect at later stages of LR development.

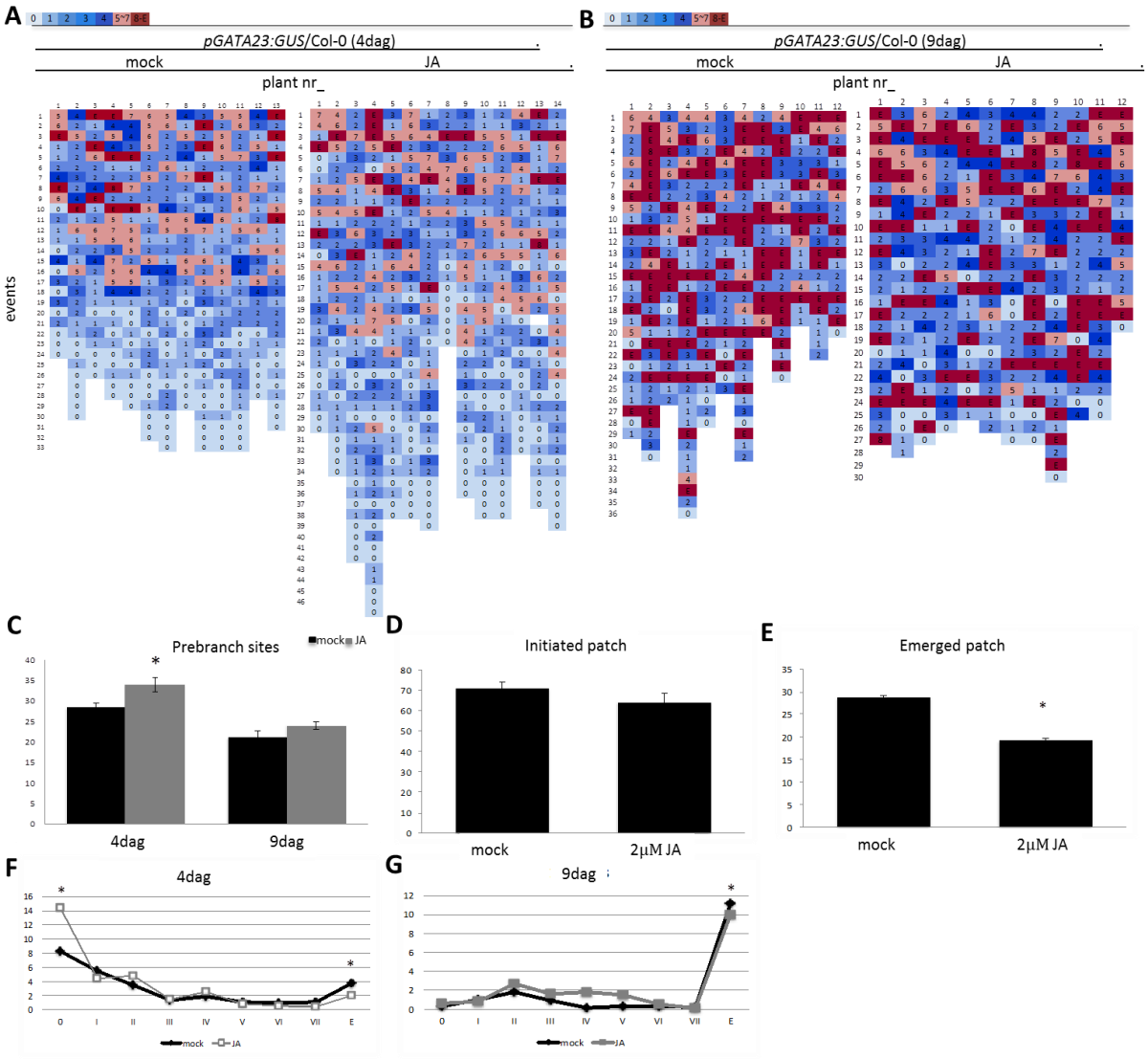


Figure 3. Effect of exogenous JA on LR development in Col-0 background. (A, B) Stages of LRP in the Col-0 background as analyzed by a method integrating *GATA23-GUS* staining and staging. Analyses were done at 4 DAG and 9 DAG. At 9 DAG, only the part of the root that was already present at 4 DAG was used for analysis. Data of one representative experiment are shown. The experiments were repeated a second time with similar results (Fig. S1). (C) Average number of total LR events (including the prebranching sites) under mock and JA treatment plants at 4 and 9 DAG. (D) Percentage of initiated patches under mock and 2-µM JA treatment. (E) Percentage of emerged patches under mock and GR24 treatments. Data in C, D and E are the averages of the two repeats. (F, G) stages of LRP at 4 DAG and 9

DAG, respectively; data of one repeat are shown. Data presented are means ± standard errors of two biological repeats ($n > 10$). *$P < 0.05$, according to the Student's *t*-test.

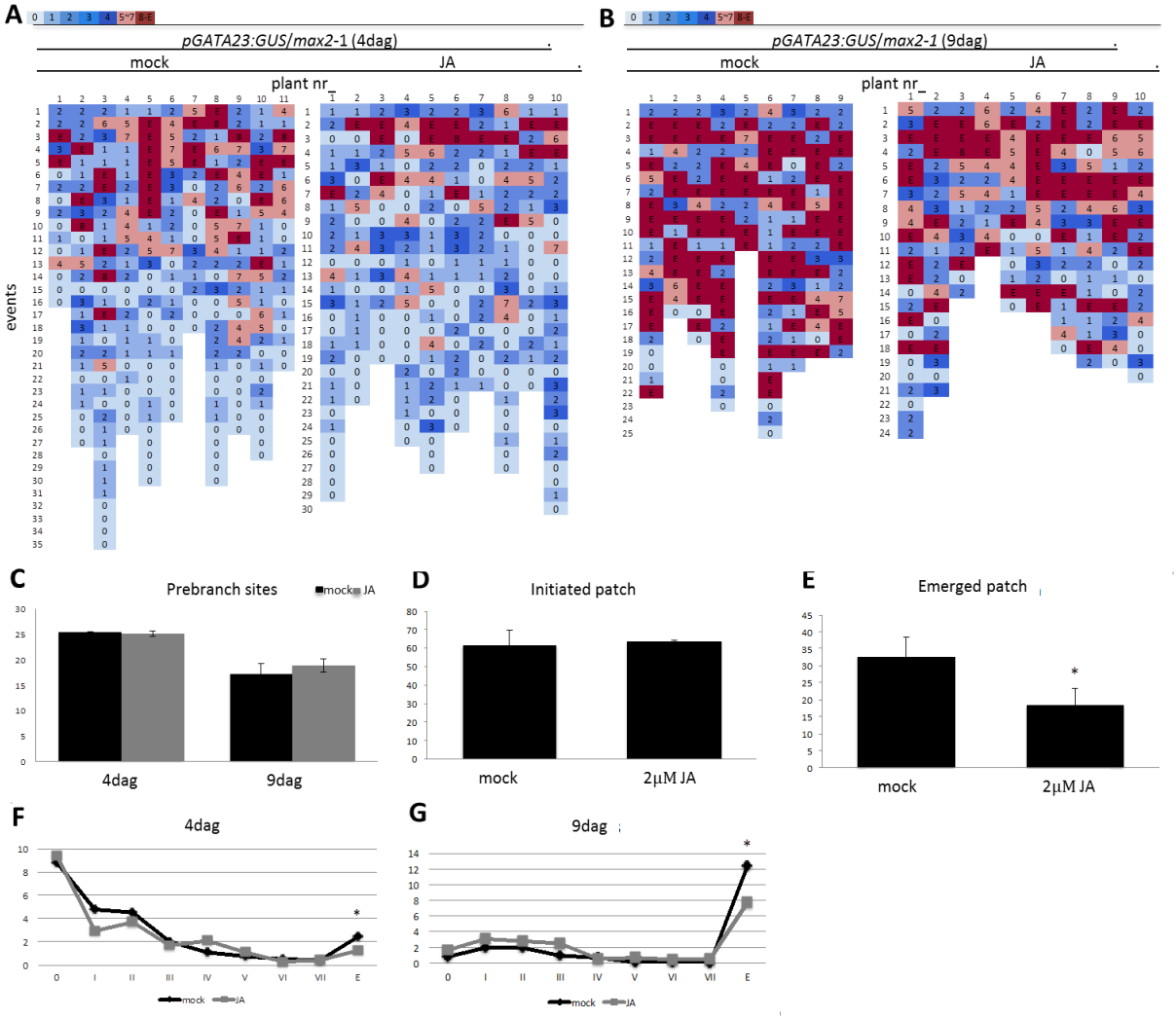


Figure 4. Effect of exogenous JA on LR development in *max2-1* background. (A, B) Stages of LRP in the *max2-1* background as analyzed by a method integrating *GATA23-GUS* staining and staging. Growth analyses were done at 4 DAG and 9 DAG on the root part that was already existing at 4 DAG. At 9 DAG, only the part of the root that was already present at 4 DAG was taken for analysis. Data of one representative experiment are shown. The experiments were repeated a second time with similar results (Fig. S2). (C) Average number of total LR events and prebranching sites under mock and JA treatment of 4- and 9-DAG plants. (D) Percentage of initiated patches under mock and 2-µM JA

treatment. (E) Percentage of emerged patches under mock and GR24 treatments. (F, G) stages of LRP at 4 and 9 DAG, respectively; data of one repeat are shown. Data presented are means ± standard errors of two biological repeats ($n > 10$). *$P < 0.05$, according to the Student's *t*-test.

Inhibition of polar auxin transport does not influence the jasmonic acid insensitivity of the SL mutants

SL mutants have been shown to have enhanced capacities of auxin transport and phenotypes to be rescued by the auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA) treatment (Bennett et al., 2006). Hence, whether addition of the auxin transport inhibitor could influence the insensitivity of the SL mutants toward JA was investigated. To this end, root lengths and LRD of WT and mutant plants were compared that were grown in the presence of JA, NPA, or a combination of JA and NPA. As shown in Figure 5, for the WT plants, application of 0.1 μM NPA did not affect primary root growth, but caused a little reduction in LRD, albeit not significant. Addition of JA reducted the main root length and increased the LRD, as previously described (Raya-Gonzalez et al., 2012). The LRD was 60% lower in plants treated with 2 μM JA together with NPA than that in mock-treated plants (Fig. 5B). However, these effects were not observed in the SL mutants. The SL-deficient and signaling mutants exhibited an insensitivity to the addition of 0.1 μM NPA and they also were insensitive to the combined treatment of NPA and JA. Considering the high auxin transport capacity in SL mutants, this insensitivity might be due to the low NPA concentration. In addition, JA was reported to influence auxin homeostasis (Sun et al., 2009) and the lack of response of SL mutants to NPA and JA might also indicate the possibility that SLs are involved in JA modulation of auxin homeostasis (Fig. 5).

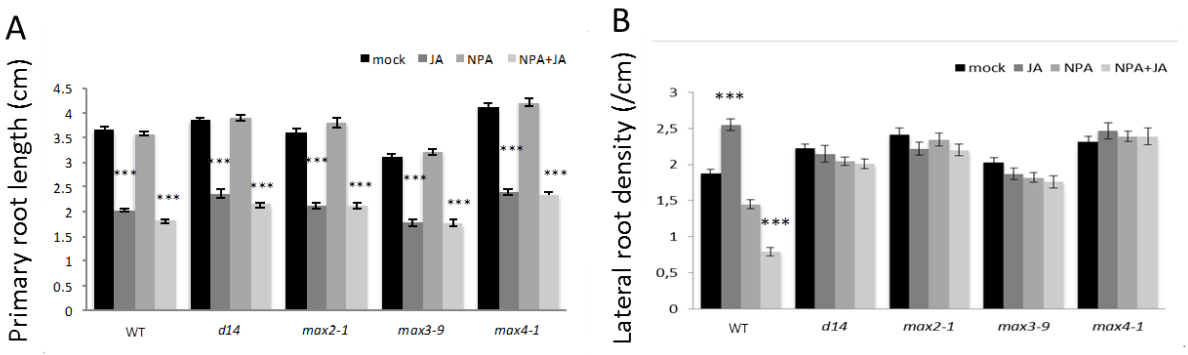


Figure 5. Effects of NPA and JA on the LR architecture of the SL mutants.

(A) Primary root length of SL mutants grown under mock treatment and in the presence JA, NPA, or a combination of NPA and JA. (B) LRD of SL mutants grown under mock treatment and in the presence JA, NPA, or a combination of NPA and JA. Comparisons were done between mock and treatment. Data represented are means ± standard errors of three biological repeats (n > 20). ***P value <0.001, according to ANOVA mixed-model statistical analyses.

Expression of strigolactone biosynthesis and signaling genes is regulated by JA

One way to explain our results is that JA modulates the expression of the SL genes to cause the effect on the LR development. To examine whether the expression of genes encoding SL biosynthesis and signaling are transcriptional or translationally regulated by JA, we investigated whether JA influenced the expression of these genes in transgenic plants containing the corresponding *pro:GUS* or *pro::GFP* fusions. Plants were grown for 5 days on 2 µM JA medium whereafter the GUS expression patterns were analyzed. Under mock conditions, the tested lines behaved as previously described (Fig. 6 and 7). *proMAX1:GUS*-derived *GUS* expression was restricted to the vascular tissue and the expression was visible as soon as it was differentiated above the differentiation zone of the meristem (Booker et al., 2005). *proMAX4:GUS*-derived *GUS* expression strongly accumulated in the root tip (Sorefan et al., 2003). Concerning the SL signaling components, expression of *proD14:GUS* was not observed in the root meristem zone,

but in the provascular strands and in the older root part and continued throughout the vascular tissue, but was also observed in the cortical cells (Chevalier et al., 2014). *proMAX2:GUS*-related staining was observed throughout the root, again restricted to the vascular tissue. GUS staining was also low in the root cap cells (Shen et al., 2007; Stirnberg et al., 2007). Special attention was paid to the expression of these genes in developing LRs. The expression of all genes was absent from the developing LRP, implying lack of SL biosynthesis or signaling genes in the young LR organ (Fig. 6 and 7).

Once the expression of the genes was established under mock conditions, the effect of JA was analyzed in plants grown for 7 days on JA. The JA treatment enhanced the GUS staining derived from *proMAX1:GUS, proD14:GUS,* and *proMAX2:GUS* expression. For *proMAX4:GUS*, no clear change in expression pattern could be observed upon JA treatment. The increase in GUS staining was remarkably high for *proD14:GUS*, because the GUS staining expanded toward the outer layers beyond vascular tissues and toward the elongation zone of the root as well as inside the LRP (Fig. 6 and 7). Taken together, these results suggest that JA regulates SL gene expression in the root.

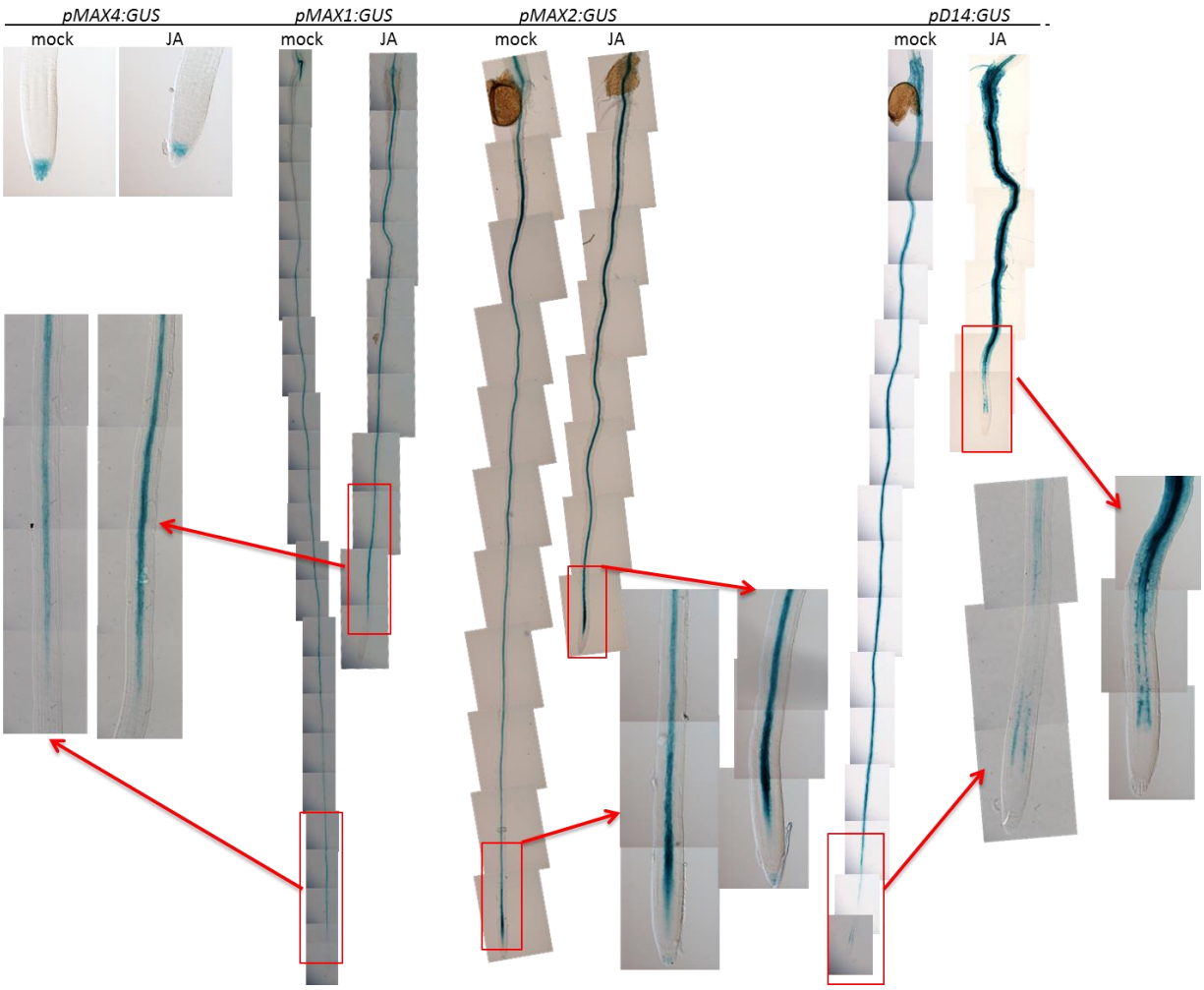


Figure 6. Overview of expression pattern of SL genes in plants treated with JA. Seedlings of each transgenic line were grown for 5 days in mock or 2 μM JA medium.

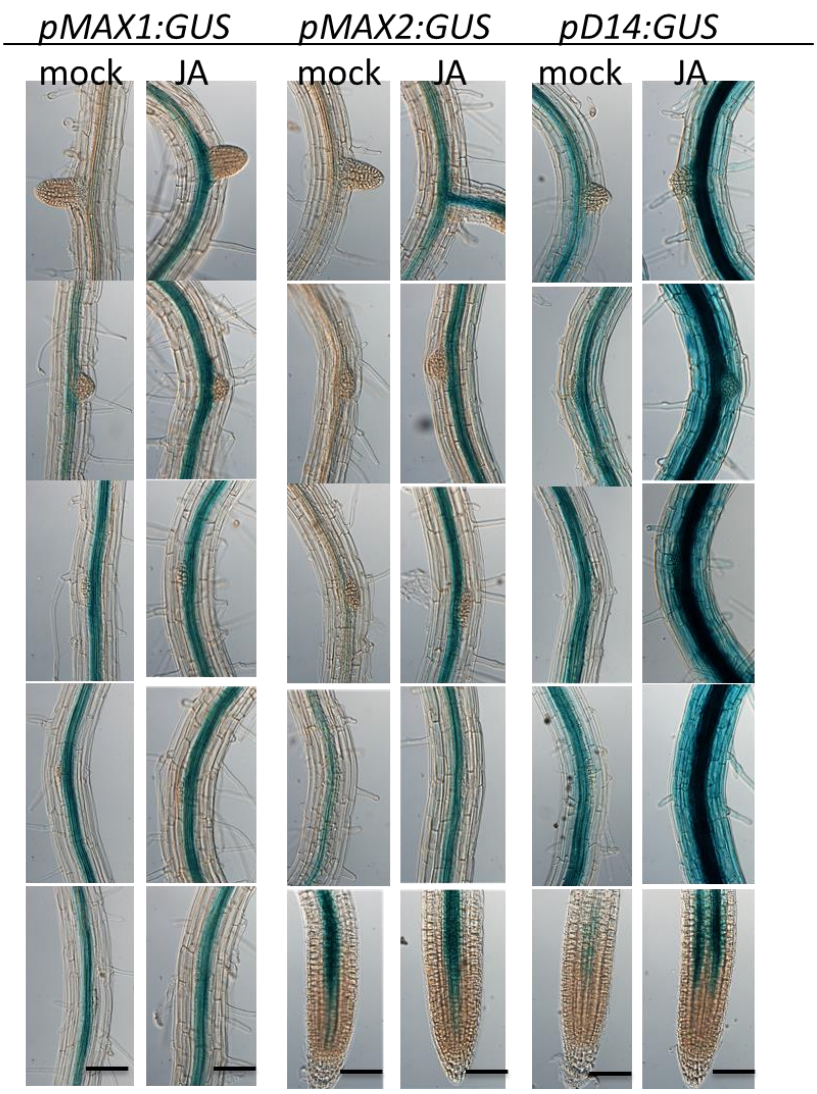
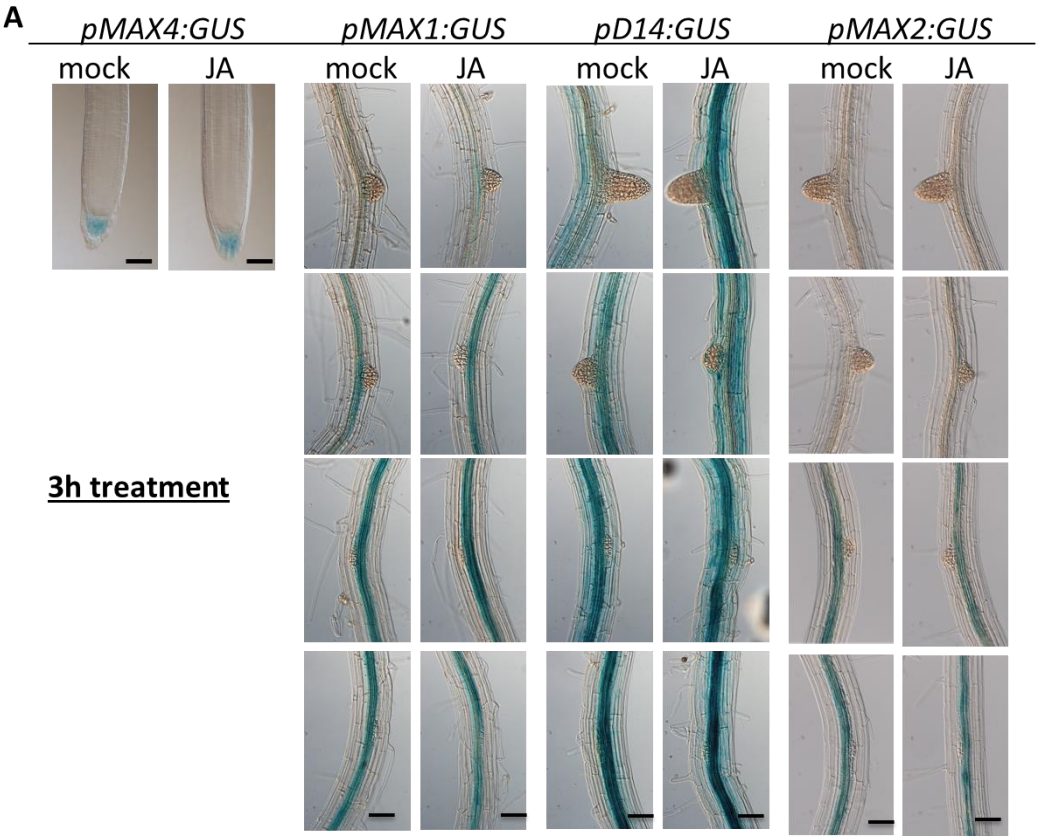


Figure 7. Expression pattern of SL genes in plants treated with JA at different stages of LR development. Scale bars = 60 µm.

Next, to study how fast the SL-related genes responded to JA, we transferred 7-day-old plants to JA medium for 3 and 24 h and analyzed the GUS staining (Fig. 8). The results showed that only the GUS staining of *proD14:GUS* was more intense after transfer for 3 h on JA-containing medium, while the expression patterns of the other genes did not differ (Fig. 8A). When plants were analyzed after 24 hours of JA treatment, besides *proD14:GUS*, also *proMAX2:GUS* plants presented an increase in GUS staining compared to mock-treated plants (Fig. 8B). The expression patterns of the two biosynthesis genes (*MAX1* and *MAX4*) did not differ during these

treatments. At these two time points, no GUS staining was observerd in the LR primordia of *proD14:GUS*. These data indicate that SL signaling is most responsive to JA and that the induction of the SL biosynthesis genes might be an indirect response.


A
pMAX4:GUS
pMAX1:GUS
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pMAX2:GUS
mock
JA
mock
JA
mock
JA
mock
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3h treatment

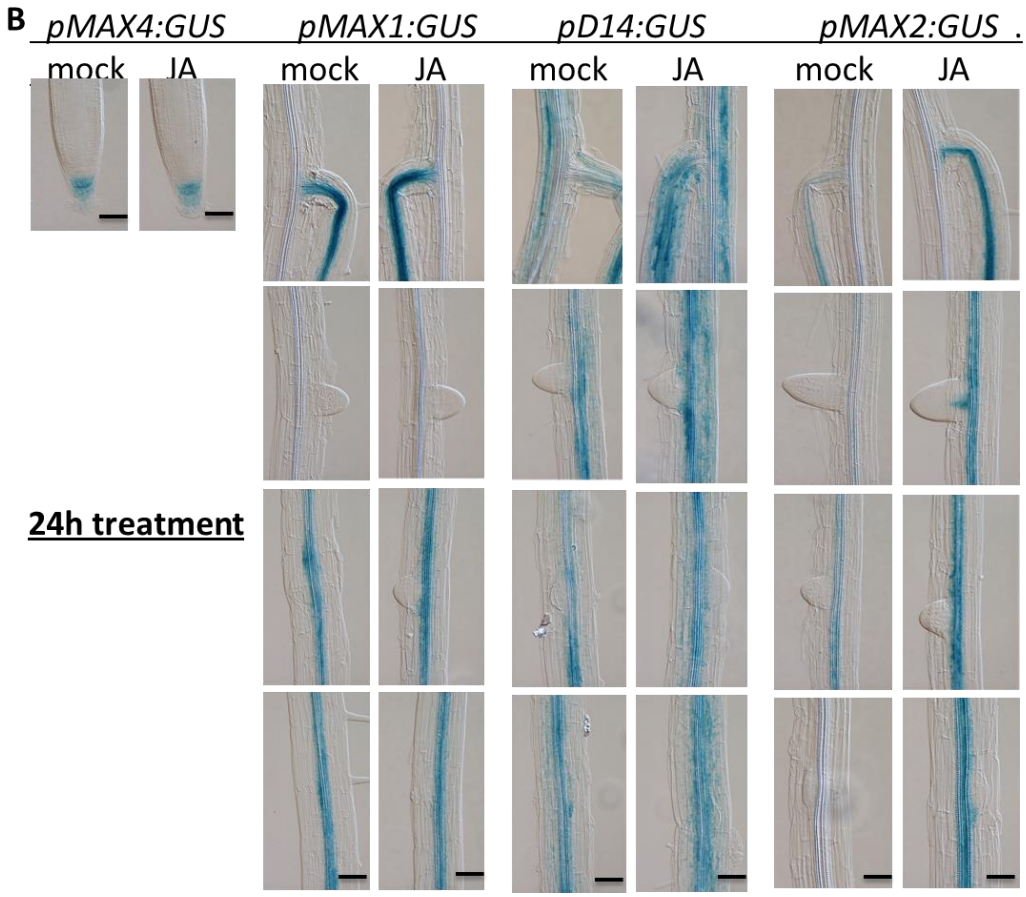

B
pMAX4:GUS
pMAX1:GUS
pD14:GUS
pMAX2:GUS .
mock
JA
mock
JA
mock
JA
mock
JA
24h treatment

Figure 8. Expression pattern of SL genes at different time points after JA treatment. Seven-day-old seedlings were transferred to JA or mock medium and roots were harvested 3 h and 24 h post treatment. For each root, pictures were taken from the shoot-root junction downward to the root meristem. (A) Expression of SL-related genes at different stages of LR development in seedlings grown under mock and JA treatment for 3 h. (B) Expression of SL-related genes at different stages of LR development in seedlings grown under mock and JA treatment for 24 h. Scale bar = 60 µm.

To confirm the influence of JA treatment on SL gene expression, quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) was executed on 5-day-old roots transferred on mock or 2 µM JA-containing medium for 3 h. *D14* and *MAX2* transcript levels were higher 3 h after JA treatment than those after the mock control (Fig. 9). No significant differences could be observed for *MAX4* or *MAX1*. Thus, the qRT-PCR data are generally in agreement with the observed *GUS* expression patterns.

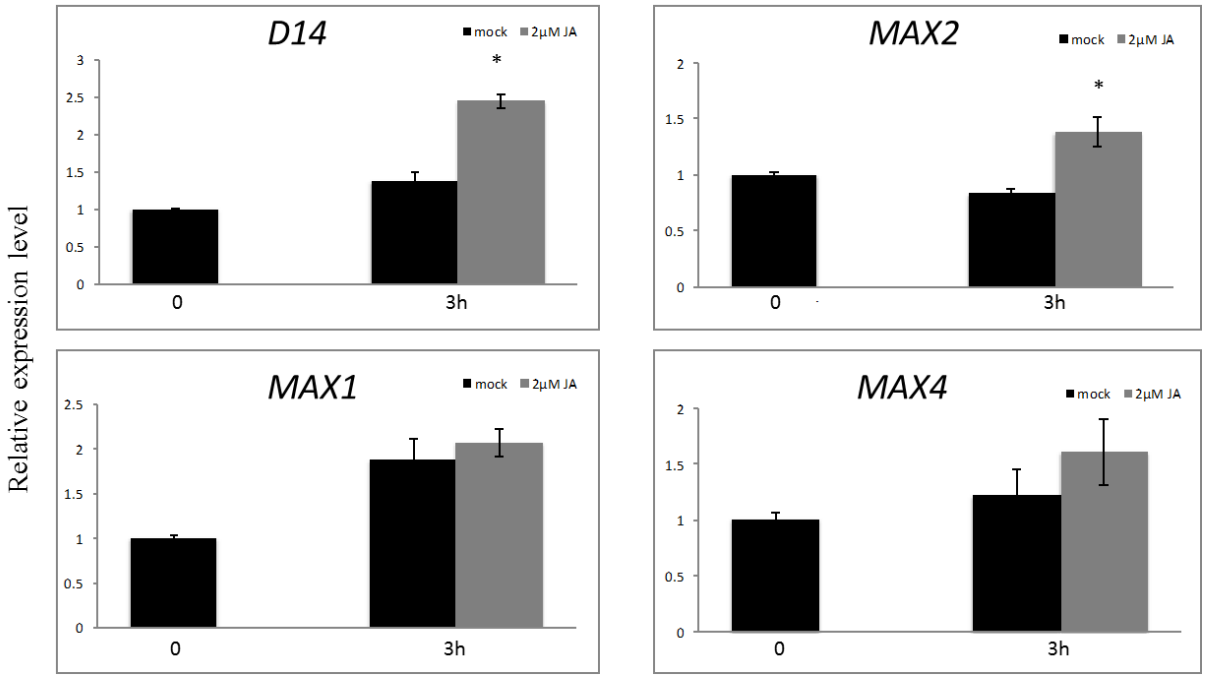
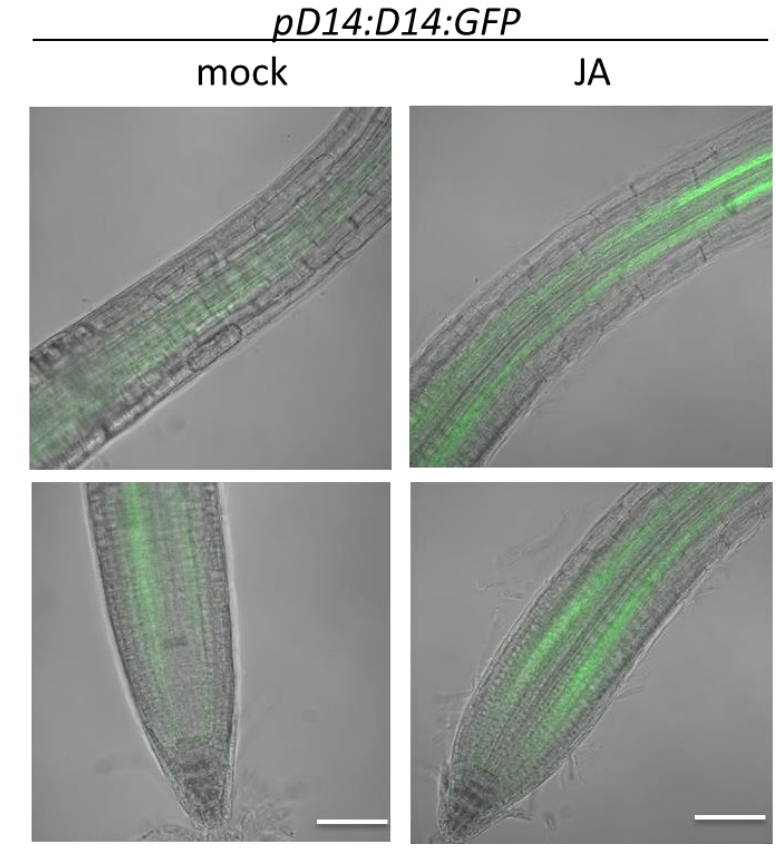


Figure 9. qRT-PCR analyses of SL-related genes in Col-0 roots upon JA treatment. Seedlings of Col-0 were grown on ½MS medium for 5 days and then transferred to medium with or without 2 µM JA. Whole roots were harvested at the moment of transfer and at 3 hours after treatment. Data presented are means ± standard errors of three biological repeats, at least 50 plants were pooled for every repeat. *$P < 0.05$, according to ANOVA mixed-model statistical analyses.

To analyze the influence of JA on the D14 and MAX2 protein level, 7-day-old seedlings of *proD14:D14:GFP* and *proMAX2:GFP:MAX2* grown on ½MS medium were transferred to medium with or without 2 µM JA. Under control conditions, D14 and MAX2 proteins were localized in the nuclei of vascular tissue cells while low fusion protien levels could be seen at the root meristem and differentiation zone (Fig. 10).

Three hours after transfer to JA medium, D14 and MAX2 protein levels were assessed by confocal microscopy. JA treatment enhanced the D14 protein expression at the root meristem and differentiation zone inside cells of the provascular tissue (Fig. 10), whereas no difference was observed in the other parts of the root (data not shown). For MAX2, an increment was also visible at and close to the meristem zone, similar to the D14 protein, but no effect was seen within other parts of the root, neither at LRP nor inside vascular tissues (Fig. 10).


pD14:D14:GFP
mock
JA

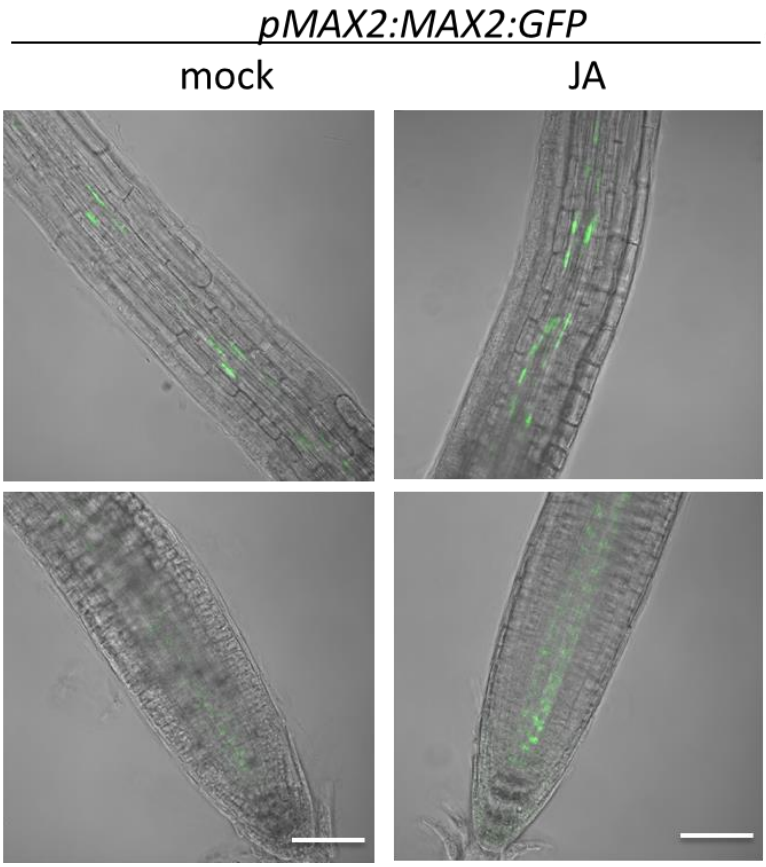

pMAX2:MAX2:GFP
mock
JA

Figure 10. Expression of SL-related genes at protein levels in Col-0 upon JA treatment. Seven-day-old seedlings were transferred to medium with or without 2 µM JA for 3 h. Whole roots were visualized and photographed with a laser scanning confocal microscope. Images shown are representatives of at least three independent experiments and three different lines, *n* = 8-12. Scale bar = 70 µm.

Influence of GR24 on mutants affected in ethylene biosynthesis or signaling

Ethylene has been shown to be involved in the GR24 impact on RH elongation (Kapulnik et al., 2011b). Moreover, studies on plant hormone crosstalk have demonstrated that ethylene regulates LR formation through interaction with auxin (Negi et al., 2008; Ivanchenko et al., 2008) and also that SLs interact with auxin to regulate LR development (Ruyter-Spira et al., 2011). Thus, to test the possibility that GR24 might act through the ethylene pathway to regulate LR formation, we examined the GR24-dependent reduction in LR formation in several ethylene signaling mutants. Conversely, we also analyzed whether the sensivity to ethylene of SL mutants differed from that of the WT for the ethylene-dependent reduction in LR formation.

First, we analyzed whether the *max* mutants had an increases sensitivity to exogenous ACC. The root length and the number of LRs were measured on 9-day-old plants grown on mock or 1 µM ACC. ACC caused a decrease in the primary root length and in the LRD of WT (Col-0) plants as previously described (Negi et al., 2008; Ivanchenko et al., 2008; Strader et al., 2010) and the same phenotype was observed for the *max* mutants (Fig. 11A and 11B). Accordingly, the LRD in *max2-1* was higher than and ACC decreased it to levels equal those in Col-0 and the other mutants.

Several ethylene mutants were investigated for the GR24-induced LRD reduction. The following mutants were used: *etr1-1* that is a dominant ethylene perception mutant (Bleecker et al., 1988), *ein2-1* with an altered ethylene signaling pathway upstream of EIN3 (Alonso et al., 1999), *ein3-1* that is affected in the early ethylene signal transduction pathway (Chao et al., 1997), and *eto1-1* that overproduces ethylene (Guzman and Ecker, 1990).

The *ein2-1*, *ein3-1*, and *etr1-1* mutants displayed a sensitivity to GR24 similar tot that of Col-0 (Fig. 11C). However, the *eto1-1* mutant, which contains increased ethylene levels (Guzman and Ecker, 1990), showed a higher sensitivity to GR24, because the LRD was to 60% lower than that of the WT (34%).

To confirm this result, we treated Col-0 plants together with 1 μM GR24 and 1 μM ACC. The combined treatment profoundly decreased the LRD, causing a 65% reduction compared to 39% caused by the GR24 treatment alone (Fig. 11E). These data suggest that ethylene and SL both repress LR formation, but, largely independently from each other.

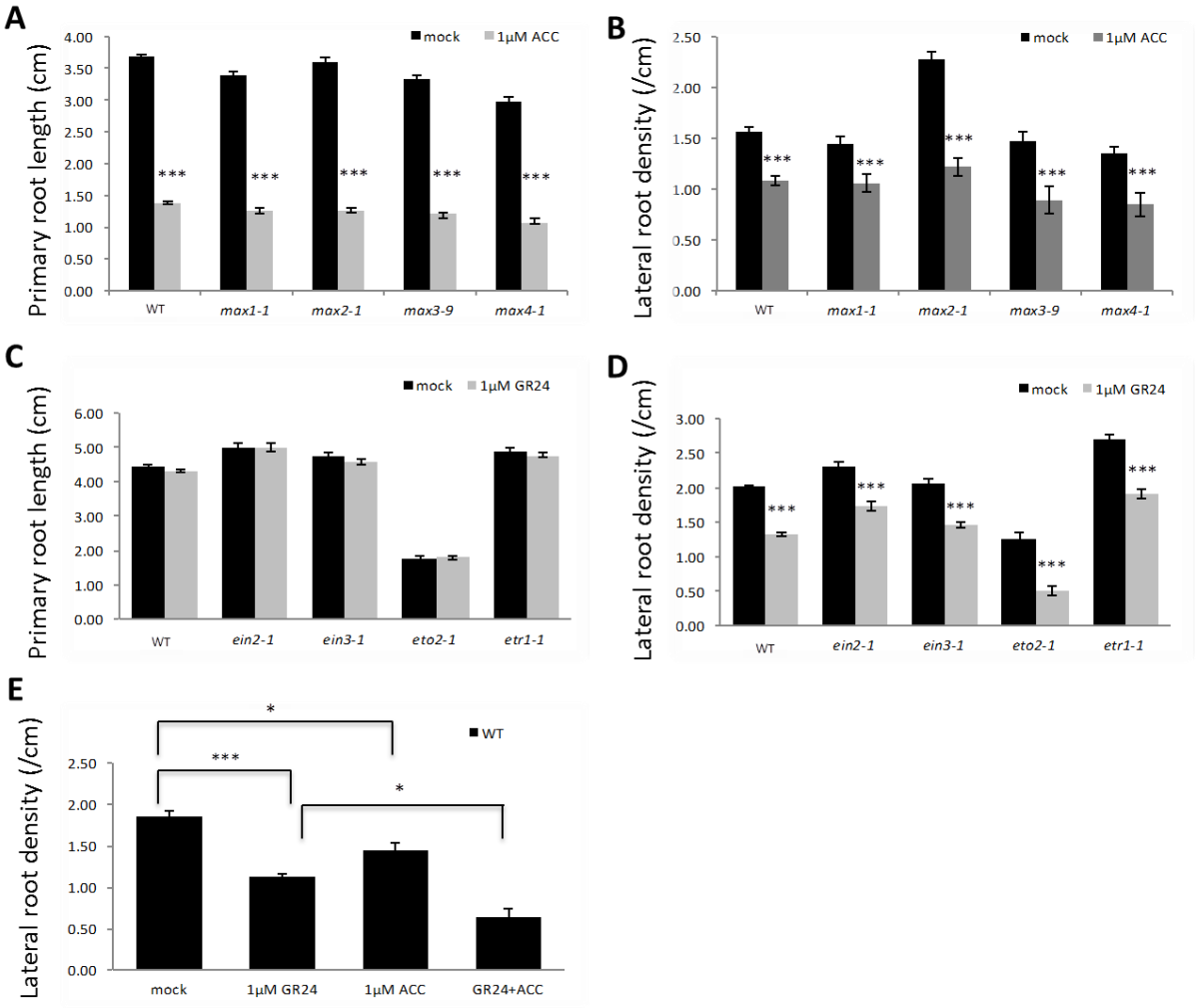


Figure 11. The interaction of ethylene with GR24 to control LR development. A. Effect of ACC on the primary root length of Col-0 and *max1-1*, *max2-1*, *max3-9,* and *max4-1*. B. Effect of ACC on LRD of the corresponding lines shown in A. C. Effect of GR24 on the primary root length of Col-0 and ethylene signaling-related mutants *ctr1-1*, *ein2-1,* and *ein3-1*, as well as *eto1-1*. D. Effect of GR24 on the LRD of the corresponding lines shown in C. (E) LRD of Col-0 upon GR24 and ACC treatment. Data presented are means ± standard errors of three biological repeats (n > 20). *P< 0.05, ***P < 0.001, according to ANOVA mixed-model statistical analyses. Comparisons were done between mock and treated plants in A-D.

DISCUSSION

Interplay of strigolactones with jasmonate on primary root and lateral root development

Phytohormones exert their effects in a complex framework of interacting responses rather than by isolated linear pathways. These networks of hormonal crosstalk can be modulated by a multitude of signals from developmental or environmental origins. The root architecture has been proposed to be finely regulated, requiring that each hormone communicates with the others, giving rise to a complex network of hormonal interactions.

Although the role of JA in plant immunity and in wound responses has been well studied, the functions in root development have been less investigated. In *Arabidopsis*, treatment with JA inhibits primary root growth, while increasing the LRD (Staswick et al., 1992; Berger et al., 1996; Raya-Gonzalez et al., 2012). Our study confirms these observations, because addition of JA decreased the primary root length by 50% compared with mock treatment in WT plants and slightly increased the LRD.

To further get insights into how JA affects LR development, we used our established system (see Materials and Methods) in which we obtain a spatiotemporal view on the LR development (see Chapter 1). These analyses revealed that in the initial developmental stages, plants respond to JA treatment by increasing the LR priming, a process during which pericycle cells become predestined to form LRs, but do not yet undergo division (De Smet et al., 2007). Later in time, these primed sites have the potential to form LRP, but not all primed events will do so (De Rybel et al., 2010). However, LR initiation did not increase, in contrast to previous results (Raya-Gonzalez et al., 2012), but the data of both studies are difficult to compare; it might well be that the increase in LR initiation described (Raya-Gonzalez et al., 2012) reflects the increase in LR

priming we observed here. As LR priming is controlled by auxin pulses within the basal root meristem zone (De Smet et al., 2007), it will be interesting in the future to unravel how JA interacts with this auxin network.

Interestingly, besides a positive effect of JA at the early stages, it redued LR outgrowth, a phenotype not described yet. Hence, JA seems to play a dual role on LR development: a stimulating one at early stages and an inhibitory one at the outgrowth level. LR priming might offer a flexible system to plants to adapt their LRD to the environmental conditions (Malamy and Ryan, 2001; Malamy, 2005; Nibau et al., 2008; Petricka et al., 2012). In that respect, JA acts as a stress hormone and it might induce the plant's potential to develop LR development, whereafter the general environmental context influences the actual number of roots that will be formed. Recently, the increase in LRD observed by exogenous JA might be due to the specific activity of the auxin biosynthesis gene *ASA1* (Sun et al., 2009). It would be interesting to test whether *ASA1* controls the JA stimulation of LR priming.

Intriguingly, JA mutants displayed a phenotype opposite to that expected from the effect of exogenous JA on LR development. Indeed, the LRD was higher in the *coi1* and *myc2myc3myc4* mutants, whereas it was lower in the *35S:MYC2* and *JAR-OX* lines with high JA signaling or (+)-7-iso-JA synthesis, respectively, than that of the WT, but did not differ in other mutants. It is difficult to explain these results, although lateral rooting could well respond differently to various JA levels or in a dissimilar manner to changes in endogenous of exogenous JA.

To explain the results, we propose a hypothesis in which lateral rooting can react to JA in a concentration-dependent-manner (Fig. 12). At low JA levels, the increase in JA biosynthesis or signaling would decrease the LR development, whereas from a certain threshold onward, the

increase in JA levels/signaling might have the opposite effect. This "threshold" might not be reachable by overexpressing JA biosynthesis or signaling endogenously through the *35S* promoter, but might only be reached by exogenous JA. It will be worthwhile to test whether this exogenous addition mimics biologically significant situations such as the JA accumulation during stress responses such as upon pathogen attack or mechanical wounding. This dose effect has been observed also for several other hormones, displaying different phenotypes depending on the genetic context or added concentrations. One example is auxin. Feeding exogenous IAA could lead to an increased root formation and the formation of epinastic cotyledons and leaves, in contrast with the auxin-overproducing *bushy1* (*bus1)* (Reintanz et al., 2001) and the allelic *supershoot* (*sps)* mutant (Tantikanjana et al., 2001) that exhibited bushy shoot formation, retarded vascularization onset, and upward curling leaves, but without changes in the root system development.

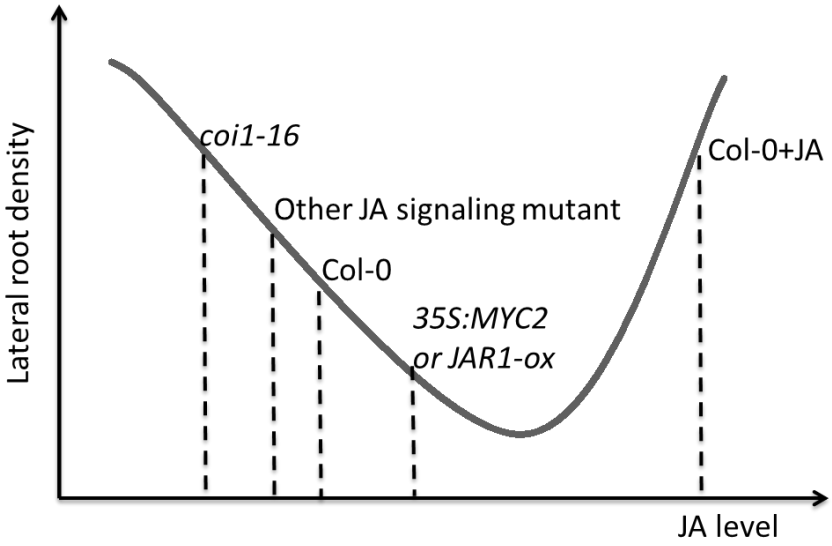


Figure 12. Model showing JA modulation of LR development. Before the threshold, endogenous JA inhibits LR development; when the concentration of JA reaches a certain level, the LR development is induced. Based on this model, the *coi1-16* mutant that has much lower JA level than Col-0 has a higher

LRD, whereas, the *35S:MYC2* transgenic line or *JAR1-OX* overexpression line inhibit LR formation. Feeding with exogenous JA would be enough to exceed the threshold and would then induces lateral root development.

Interestingly, the increase in LRD observed by exogenous JA was not seen in any of the SL biosynthesis or signaling mutants, indicating that SLs play an important role in the effect of JA on LR development. In contrast, SLs did not influence the JA impact on the root length. This observation supports the hypothesis that the influence on lateral rooting by JA happens via different regulatory pathways compared to the effect on the main root. Furthermore, the JA effect on LR priming seems to be controlled in a different manner from that on the emergence. Indeed, *max2-1* mutants still displayed the JA-dependent reduction in LR emergence, but they were not affected in the JA-dependent increase in LR priming. These results suggest that SL interplays with JA at a very early stage of LR formation, a step occurring in the basal root meristem zone.

LR initiation is mainly regulated by auxin transport and signaling and auxin synthesis induction has been shown to play a major role in JA-induced lateral rooting (Dubrovsky et al., 2008; Sun et al., 2009; De Smet et al., 2012; Cai et al., 2014). Additionally, increased auxin transport capacity is a common phenotype for the SL biosynthesis and signaling mutants (Bennett et al., 2006). Interestingly, reducing the auxin transport by the addition of NPA did not rescue the phenotype of SL mutants to the WT level. Considering the auxin transport capacity is higher in SL mutants than that of the WT, 0.1 μM NPA might not have been enough to change the phenotype. JA has been reported to regulate the transcriptional expression of the auxin biosynthesis gene *ASA1* and to influence the endocytosis of the auxin efflux transporter PIN2 in roots (Sun et al., 2009; Sun et al., 2011). Thus, the changes in polar auxin transport activity in the SL mutants might still

deregulate the JA-induced changes in the PIN localization required for the effects on LR development. In the future, PIN1, PIN2, PIN3, and PIN7 protein levels and polarities upon JA treatment should be tested in the SL mutants in roots.

Our data support a direct involvement of SLs, via the JA-dependent induction of the SL signaling genes, on the JA impact on LR priming. Indeed, qRT-PCR and transcriptional and translational fusions revealed that JA treatments induced the *D14* and *MAX2* gene and protein levels. This induction happened within 3 h after treatment and was more pronounced for *D14* than for *MAX2*. At later stages, in plants treated for several days with JA, also the *MAX1* transcription increased. Interestingly, JA treatments increased both D14 and MAX2 within the basal root meristem zone where LR priming occurs (De Smet et al., 2007). Hence, JA-induced *D14* and *MAX2* expression might contribute to the impact of JA on LR priming, but not to the reduced LR emergence, in agreement with the phenotype of the *max2-1* mutant.

We also addressed the question whether JA biosynthesis or signaling might interrelate with the effect of GR24 on the LRD. However, JA biosynthesis or signaling did not interact, although mutants and transgenic lines with modulated levels of the transcription factor MYC2 or its homologs displayed a reduced sensitivity to GR24. MYC2 is a central transcription factor controlled by JA, but also known as a central hub that controls the interaction with many other phytohormones to regulate various processes, such as photomorphogenesis (Robson et al., 2010; Hong et al., 2012; Kazan and Manners, 2013). SLs act in close interrelation with the light signaling pathways and have been shown to be involved in photomorphogenesis (Shen et al., 2007; Mayzlish-Gati et al., 2010; Tsuchiya et al., 2010; Koltai et al., 2011). Hence, the modified GR24 sensitivity of *myc2* mutants or transgenic lines might rather confirm this close interrelation than an intimate crosstalk with JA.

Ethylene often works together with JA to control stress responses (Lorenzo et al., 2003; Kazan, 2015). However, no clear interaction between ethylene and SL was found here, although ethylene, just like SLs, negatively regulates LR formation via regulation of auxin efflux transporters (Ivanchenko et al., 2008; Negi et al., 2008; Negi et al., 2010; Lewis et al., 2011). The only phenotypic change we observed was that endogenously or exogenously increased levels of ethylene enhanced the sensitivity to GR24, but this phenomenon needs to be investigated. Interestingly, ethylene has been shown to play a major role in the GR24-dependent increase in RH elongation. Thus, the impact of SLs on LR development as well as on RH elongation, both root phenotypes, happens through different molecular pathways.

In summary, we have demonstrated that JA causes an opposite role in LR priming and LR emergence and that the induction of SL signaling seems to play a main role in the JA effect on LR priming. What happens downstream of the SL signaling pathway, such as modulation of the PIN accumulation at the membrane, needs to be established and is worthwhile testing in the future.

MATERIALS AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* (L.) Heynh. plants used in this study were of the Columbia-0 (Col-0) accession. The plant material used had been described previously: *coi1-16* (Devoto et al., 2002), *jai3-1* (Chini et al., 2007), *ninja* (Acosta et al., 2013), *jin1-2* (Lorenzo et al., 2004), *35S:MYC2* (Lorenzo et al., 2004), *myc2myc3myc4* (Fernandez-Calvo et al., 2011), *JAR1-OX* (Gutierrez et

al., 2012), *max1-1* (Stirnberg et al., 2002), *max3-9* (Booker et al., 2004), *max4-1* (Sorefan et al., 2003), *max2-1* (Stirnberg et al., 2002), *d14* (Nakamura et al., 2013), *etr1-1* (Bleecker et al.,1988), *ein2-1* (Alonso et al., 1999), *ein3-1* (Chao et al., 1997), *eto2-1* (Guzman and Ecker, 1990), *proMAX1:GUS* (Booker et al., 2005), *proMAX2:GUS* (Stirnberg et al., 2007), and *proMAX4:GUS* (Sorefan et al., 2003). The *proD14:GUS* lines were constructed and the primers used were:

Forward; GGGGACAACTTTGTATAGAAAAGTTGCTAAGAGTTCGTCTTGAGAGGAGC

Reverse: GGGGACTGCTTTTTTGTACAAACTTGCTTTTTTATGTGTTTGGGTTT.

Seeds were surface-sterilized with 70% (v/v) ethanol, 0.05% (v/v) sodium dodecyl sulfate solution for 5 min, then washed with 95% (v/v) ethanol for 5 min, and sown on sterile plates containing half-strength Murashige and Skoog (½ MS) medium supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar. Plants were stratified at 4°C for 2 days in the dark, then transferred to a growth chamber at 21°C with a 16-h light/8-h dark photoperiod. GR24 or JA (Sigma-Aldrich) was supplemented to the growth medium before solidification and plants were grown for the indicated time. Experiments were repeated three times. Chemical compounds were added in the following concentrations: 1 µM GR24, 2 µM JA, 1 µM ACC, and 0.1 µM NPA.

Analysis of root architecture traits

Primary root length was measured on digital images of the plates with ImageJ software (http://rsb.info.nih.gov/ij/). The number of emerged LRs was counted with a binocular microscope (Leica S4E). Clearing of the tissues and classification of the developmental stages of LRP were according to Malamy and Benfey (1997). Experiments were repeated three times, means of replicates were subjected to statistical analysis by ANOVA (SAS Institute).

Stage determination by *GATA23* expression analysis

Seeds of *pGATA23:NLS-GFP-GUS* (in Col-0 or *max2-1* background) (De Rybel et al., 2010) were sown on square Petri dishes containing ½MS medium with 1% (w/v) sucrose supplemented with 2 µM JA or with same volume of acetone as control and were placed at 4°C for 2 days in the dark. Seedlings were grown in a vertical position under 16-h light/8-h dark condition at 21°C. At 4 DAG, half of the seedlings were harvested for analysis. For the remaining seedlings, the position of the main root tip was marked and the plates were transferred back to the growth room. At 9 DAG, the root parts above the mark were harvested. Samples were stained with GUS as described below and then cleared according to Malamy and Benfey (1997). Samples were finally analyzed under a microscope (see below). For the calculation of the percentage of initiated patches, the total of initiations at 9 DAG was divided by the total of events present at 4 DAG. Similarly, for the calculation of the percentage of emerged patches, the total emerged LRs at 9 DAG was divided by the total events present at 4 DAG. Experiments were done twice and the average values were subjected to statistical analysis by the Student' *t*-test

Histochemical analysis

Complete seedlings were stained in multiwell plates as described (Jefferson et al., 1987): seedlings were sequentially incubated in (i) 90% (v/v) acetone (4°C); (ii) NT buffer (100 mM Tris; 50 mM NaCl); (iii) ferricyanide solution (2 mM K3[Fe(CN)6] in NT buffer); (iv) assay solution (2.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Thermo Scientific) in ferricyanide solution); each step (from i to iii) was done for 30 min, step iv was done according to the gene expression: *proD14:GUS* for 2 h, *proMAX2:GUS* for 3 h, *proMAX1:GUS* and

proMAX4:GUS overnight. The staining was stopped by washing the seedlings in NT buffer. Samples were mounted in 50% (v/v) glycerol and were observed and photographed by a differential interference contrast microscope (Olympus BX510). Alternatively, samples were directly mounted in chloral hydrate solution (chloral hydrate:water:glycerol, 8:3:1) and microscopically analyzed as described below.

Microscopy

For confocal microscopy images, the Zeiss LSM5 Exiter or Olympus FV10 ASW confocal scanning microscopes were used. Approximately 12 seedlings/images were examined and at least three independent experiments were done.

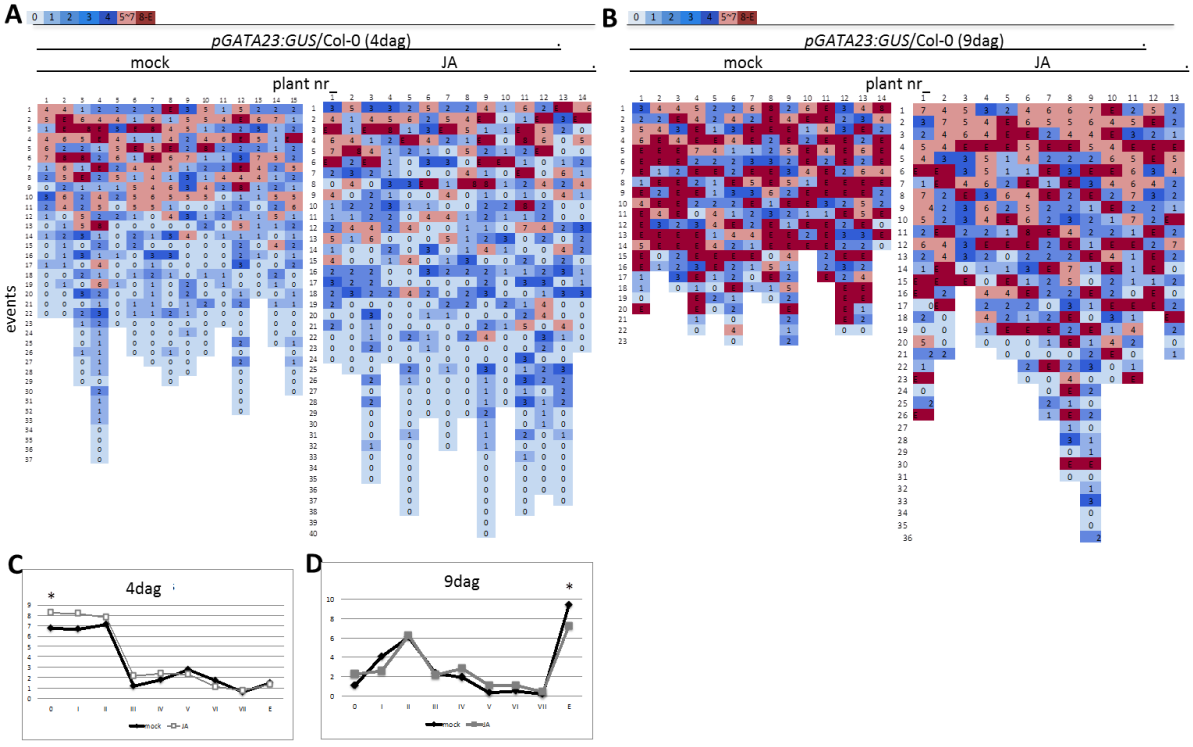
RNA isolation, qRT-PCR, and statistical analysis

Arabidopsis Col-0 WT seeds were sown in ½MS medium on a sterile 20-µm wide nylon mesh (Prosep) for easy transfer. Seeds were stratified for 2 days at 4°C and then grown in vertical position under long-day condition (16-h light/8-h dark) at 21°C. After 5 days, seedlings were transferred to fresh medium supplemented with 2 µM JA or with the same volume of 100% EtOH as control. Root material was harvested at 0, 3, and 24 h after transfer to new plates and flash-frozen in liquid nitrogen. Approximately 100 seedlings were used for each treatment at each time point and the experiment was repeated three times.

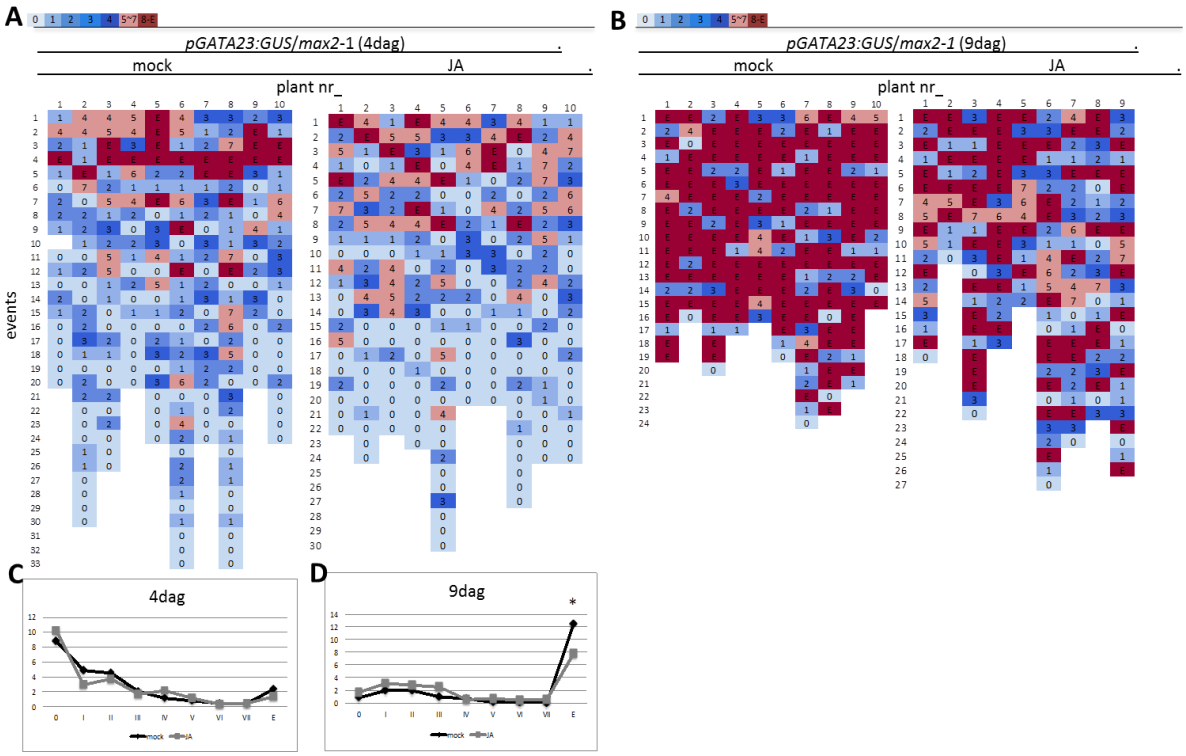
Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Genomic DNA was removed by DNase treatment, and the RNA samples were purified through NH_4Ac (final concentration of 2.5 M) precipitation. Samples were

quality-controlled and quantified with a Nano-Drop Spectrophotometer (Isogen). One microgram of RNA was reverse-transcribed into cDNA with the iScript cDNA synthesis kit (BioRad) and subsequently diluted 25 times. Real-time qRT–PCR was done on a LightCycler 480 (Roche Diagnostics) with SYBR Green for detection, in triplicate on a 384-multiwell plate, in a total volume of 5 µL and cDNA fraction of 10%. Cycle threshold (Ct) values were obtained with the accompanying software and analyzed with the 2–ΔΔCT method (Livak and Schmittgen, 2001). The values were normalized against those of ACTIN2 (ACT2, AT3G18780) that was used as an internal standard. Statistical analysis of expression profiling were done as described (Rasmussen et al., 2013b).

Supplemental Data



Supplemental Figure 1. Effect of exogenous JA on LR development in Col-0 background. (A, B) Stages of LRP in the Col-0 background. Analysis method as described in the text. (C, D) Stages of LRP at 4 and 9 DAG, respectively.



Supplemental Figure 2. Effect of exogenous JA on LR development in *max2-1* background. (A, B) Stages of LRP in the *max2-1* background. (C, D) Stages of LRP at 4 and 9 DAG, respectively.

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Chapter IV

Identification of *SMXL* genes involved in the effect of strigolactones on root architecture

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Author contributions: LX.J contributed to Fig 1, 3-6 and wrote the chapter, S.S and AD.K contributed to Fig 7, C.M contributed to Fig 2, B.M, S.S and S.G read and commented on the manuscript.

ABSTRACT

Strigolactones (SLs) are carotenoid-derived plant hormones that regulate many aspects of plant development. SL perception involves an ubiquitin-mediated signaling system in which the suppressor of kinetochore protein1 (SKP1), Cullin (CUL) and F-box protein (SCF) E3 ligase MORE AXILLARY GROWTH2 (MAX2) plays an essential role. Signaling downstream of MAX2 is still poorly defined. Recently, two proteins, DWARF53 in rice (*Oryza sativa*) and SUPPRESSOR OF MAX2 1 (SMAX1) in *Arabidopsis thaliana*, have been discovered to be targets of the SCF^{MAX2} complex. However, because *smax1* mutants do not reverse the lateral root formation phenotype of *max2*, one or more SMAX1 homologs have been proposed to act downstream of MAX2 to control root architecture. Here we showed that *SMXL2* and *SMXL7*, homologs of *SMAX1*, were induced by SL at the transcriptional level in the root and that SMXL7 stability is controlled by SLs. Furthermore expression analysis revealed that *SMXL2* transcripts were more abundant in the vasculature of the young part of the main root, where the emerging lateral root primordia are located, whereas *SMXL7* transcripts were located preferentially in the hypocotyl and in the old part of the main root, until the first lateral root emerged. These differential expression patterns indicate that SMXL2 and SMXL7 might have varying SL-related functions in the root.

INTRODUCTION

Strigolactones (SLs) are carotenoid-derived plant hormones regulating many aspects of plant development. SLs have been implicated in the control of shoot branching, secondary stem growth lateral root formation, root hair elongation, primary root growth, adventitious root initiation, and senescence in *Arabidopsis*, tomato (*Solanum lycopersicum*), pea (*Pisum sativum*),

and petunia (*Petunia hybrida*) (Woo et al., 2001; Snowden et al., 2005; Li et al., 2009; Agusti et al., 2011; Kapulnik et al., 2011a, 2011b; Koltai, 2011; Ruyter-Spira et al., 2011; Hamiaux et al., 2012; Rasmussen et al., 2012a, 2012b).

Recently, progress has been made in understanding of the SL signaling pathway. One of the α/β-hydrolase superfamily proteins, DWARF14 (D14), has been shown to be a SL receptor. *d14* mutants in several species are insensitive to SLs (Kagiyama et al., 2013; Waters et al., 2012; Hamiaux et al., 2012), and the rice (*Oryza sativa*) D14 protein and its ortholog in petunia, DAD2, have been shown to bind and hydrolyze the biologically active SL analog GR24 (Hamiaux et al., 2012; Kagiyama et al., 2013; Nakamura et al., 2013). Another important component of the SL signaling complex is an F-box protein, MAX2/D3/RMS4 (Stirnberg et al., 2002; Ishikawa et al., 2005; Johnson et al., 2006) that is linked to an SCF complex, catalyzing the ubiquitination of proteins assigned for proteasomal degradation (Moon et al., 2004). Similar to other plant hormone signal transduction pathways, the D14-SCF^{MAX2} complex is expected to ubiquitinate proteins to target them for proteasomal degradation, a step that results in the activation of the SL-responsive pathways. A breakthrough in identifying the target proteins of the SCF^{MAX2} has been made with the discovery of D53 in rice (Jiang et al., 2013; Zhou et al., 2013). D53 is a class I Clp ATPase protein that acts as a SL signaling repressor. The *d53* dominant mutants display a dwarf and high-tillering phenotype in rice and are insensitive to GR24 (Jiang et al., 2013; Zhou et al., 2013). Additionally, D14 interacts with D3 and then recruits D53 into the complex whereafter it gets ubiquitinated and degraded by the proteasome.

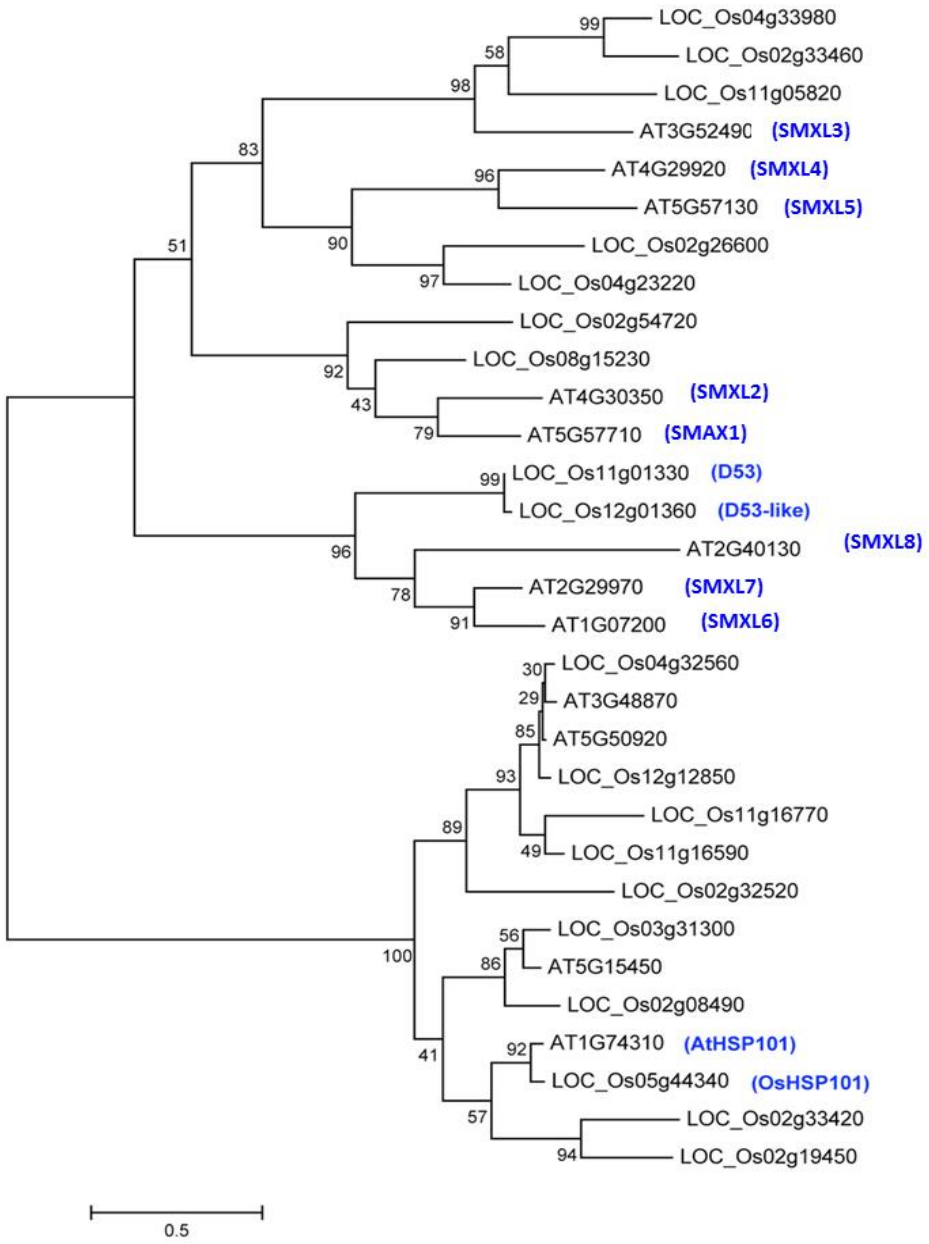

Figure 1. Phylogenetic tree of D53-like and SMAX1 family proteins in rice and *Arabidopsis*. Figure adapted from Jiang et al., 2013.

In *Arabidopsis*, a D53 homolog, SMAX1 (Fig. 1), was discovered as a suppressor of *max2* mutants (Stanga et al., 2013). The *smax1* mutants overcome the seed dormancy and seedling photomorphogenesis phenotypes of *max2*, but do not reverse either the lateral root formation or the branching phenotype. *SMAX1* belongs to an eight-gene family in *Arabidopsis* (Fig. 1). *SMAX1* together with seven other homologs, designated *SMAX1-LIKE* (*SMXL*), are differentially

expressed in various *Arabidopsis* tissues. For example, based on quantitative reverse-transcription-polymerase chain reaction (qRT-PCR), *SMAX1* is abundantly expressed in seeds, seedlings, leaves, and senescent leaves and *SMXL7* in axillary shoots and senescent leaves, whereas *SMXL3* transcription level is predominant in the root (Stanga et al., 2013). Furthermore, *max2smxl* mutants respond to GR24, indicating that the function of SMAX1 is redundant in SL signaling. Together, the SMXL family members have been hypothesized to each play a distinct role in plant development and each to be targeted for degradation by SL signaling to activate specific responses (Stanga et al., 2013). We investigated the role of SMXL proteins in lateral root development by means of an expression analysis together with a mutant analysis.

RESULTS

SMAX1, SMXL2 and *SMXL7* are transcriptionally induced by GR24 in the root.

To select proteins potentially involved in SL-dependent root responses, we did a survey of the *SMXL* genes that were upregulated by GR24 in a *MAX2*-dependent manner. To this end, we scanned the RNAseq data available in the laboratory on whole roots treated or not for 6 h with 1 µM GR24. We found that *SMXL2* and *SMXL7* were upregulated by GR24 treatment, whereas *SMAX1*, *SMXL6*, and *SMXL7* that were downregulated in *max2-1* mutants compared to the wild type (WT) were selected for further analysis. Based on the protein sequence similarity, the SMXL family could be divided into two large groups: *SMXL1* and *SMXL2* belong to the same group and SMXL7 to a different group (Stanga et al., 2013; Fig. 1).

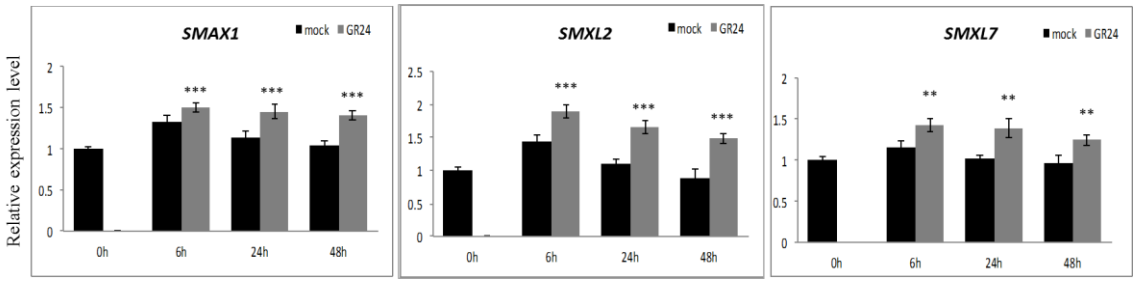

Figure 2: Relative transcriptional levels of *SMXL1*, *SMXL2,* and *SMXL7* in the presence of GR24 at different time points. Data presented are means ± standard errors of three biological repeats (*n*>100. ***P* < 0.01, ****P* < 0.001, according to ANOVA mixed-model statistical analysis.

Firstly, the expression of *SMAX1*, *SMXL2*, and *SMXL7* was reconfirmed by qRT-PCR. Plants were grown for 7 days, transferred to mock or 1 µM GR24-containing media, and at different time points, the whole roots were harvested for analysis. The three genes were induced upon GR24 treatment already 6 h after treatment and, compared to the mock treatment, they were upregulated until 48 h after treatment, the last investigated time point (Fig. 2). Although significant, the induction levels were rather small. The expression of *SMAX1* was induced as expected from 6 h and kept increasing at later hours after treatment. All these data suggest that *SMAX1*, *SMXL2*, and *SMXL7* are responsive to SL.

Secondly, we wanted to investigate the spatial expression patterns of *SMAX1*, *SMXL2*, and *SMXL7*. Therefore, we constructed the *proSMAX1:GUS*, *proSMXL2:GUS,* and *proSMXL7:GUS* reporter lines with approximately 3 kb promoter region upstream of the ATG start codon of these genes and used these constructs to transform WT *Arabidopsis* (see Materials and Methods). The generation of the *proSMAX1:GUS* lines is still in progress and the data are not available yet. Homozygous lines for the other two *pro:GUS* lines were grown for 7 days in the presence and absence of GR24 and the expression was analyzed by the histochemical GUS assay. In total, two independent homozygous lines for *proSMXL2:GUS* and three for *proSMXL7:GUS* were

analyzed (Fig. 3 and 4). For each line, at least eight plants from 10 were found with the same expression pattern.

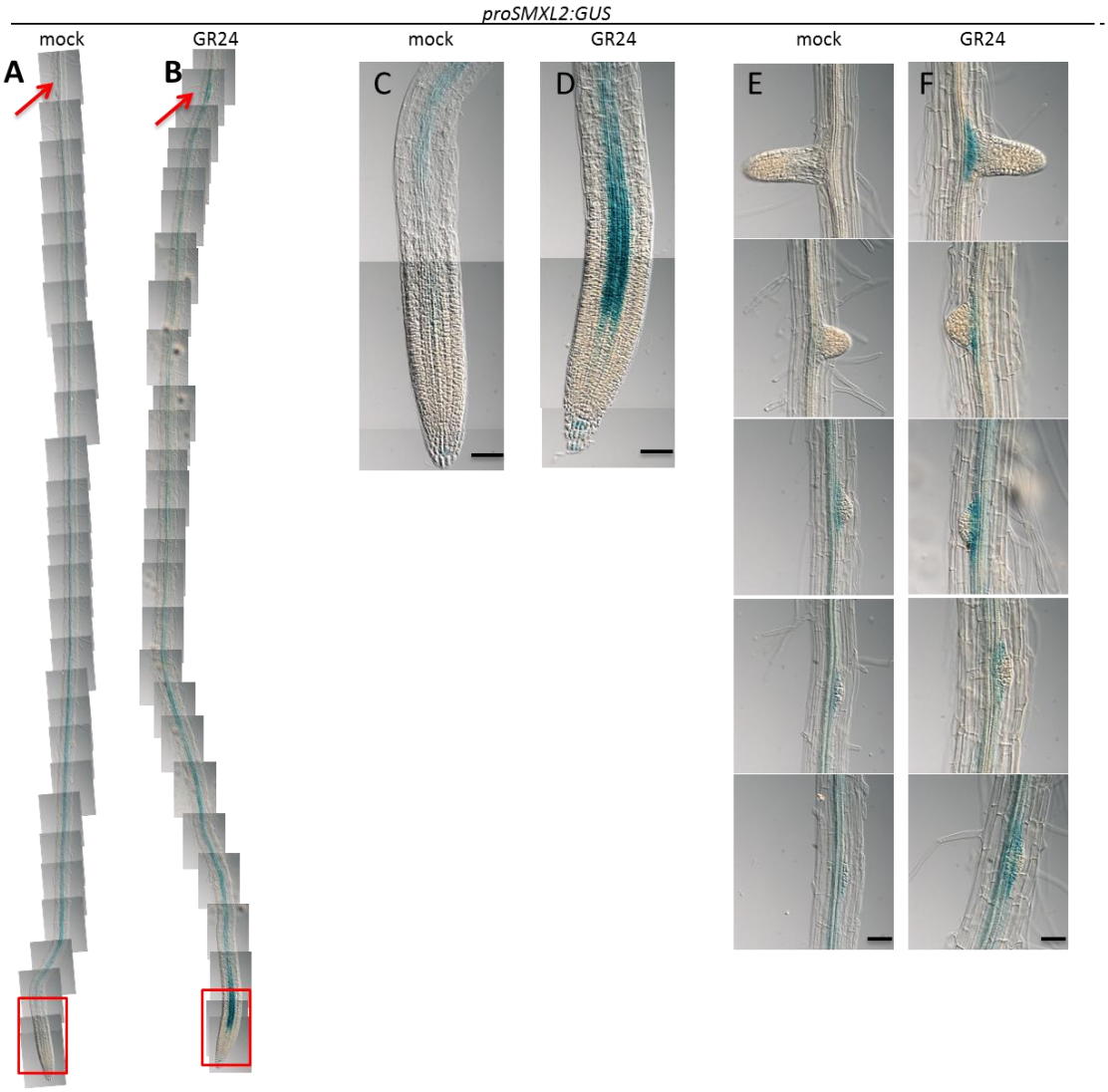

Figure 3. Expression pattern of *SMXL2* in the root and upon GR24 treatment. Seeds of each line were sown on medium with and without 1 µM GR24 for 7 days. (A, B) Overview of *proSMXL2:GUS* expression in the root with and without GR24 treatment, respectively. Arrows indicate lateral root primodia that do not emerge. (C, D) Magnification of the framed regions in (A) and (B), respectively (scale bar = 50 µm). (E, F) Stages of lateral root primordia of *proSMXL2:GUS* plant with and without GR24 treatment, respectively. Scale bar =50 µm. Arrows indicate lateral root primordia that do not emerge. *n* = 8-12 per repeat.

After 7 days of growing in mock medium, the GUS activity of the *proSMXL2:GUS* line was restricted to the vascular cylinder and this expression pattern was only seen in the young part of the root, starting from the main root part with young lateral root primordia that had not yet emerged, downward to the transition zone of the main root (Fig. 3). No or very low GUS staining was found in the root apical meristem, whereas a very weak GUS activity was observed in the root cap. In the leaves, blue GUS staining was also visible (data not shown). The expression pattern of *SMXL2* is similar that described in www.arabidopsis.org (Fig. S1), suggesting that the lines produced in our laboratory represent the actual expression profile. GR24 treatment enhanced the GUS staining, especially in the vascular tissue of the main root at the basis of the emerging lateral roots and within the meristematic zone of the main root, as well as in the root cap (Fig. 3).

The expression pattern of the *proSMXL7:GUS* plants (Fig. 4) had a lower GUS activity than that of the *proSMXL2:GUS* plants. The GUS staining was especially seen in the vascular tissue of the shoot and the hypocotyl. The expression in the root was very low and could only be detected in the vasculature of the older part of the root until the first lateral root emerged. This expression pattern is also in agreement with the data described in www.arabidopsis.org (Fig. S2). When grown on GR24, GUS staining was enhanced in the vascular tissue at all locations, also within the root.

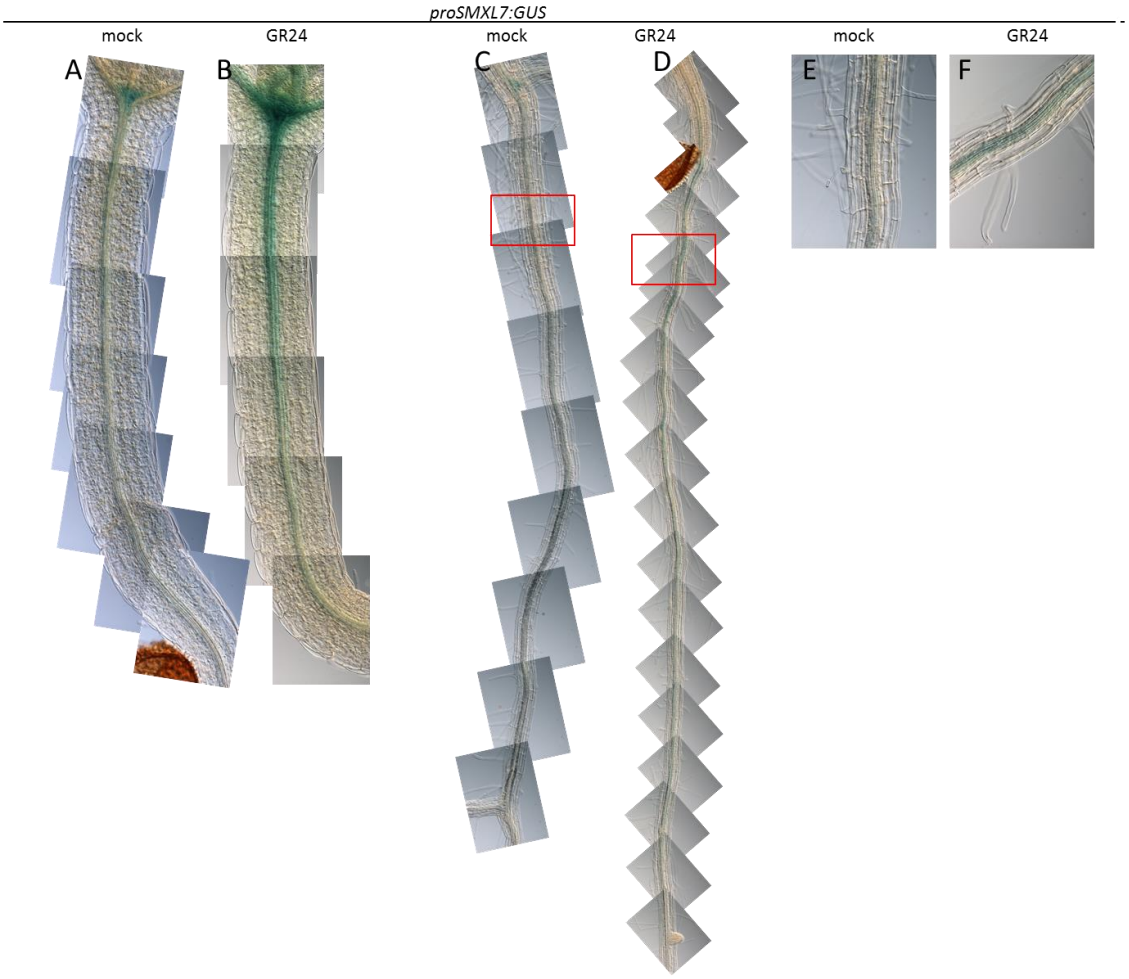

Figure 4. Expression pattern of *SMXL7* in the root and upon GR24 treatment. (A, B) Overview of hypocotyl of a *proSMXL7:GUS* plant with and without GR24, respectively. (C, D) Overview of the *proSMXL7:GUS* expression in the primary root with or without GR24. (E, F) Magnifications of the framed regions in (C, D); respectively. Three independent lines with three independent repeats were investigated. *n* = 8-12 per repeat.

Thirdly, to investigate how fast these two genes respond to GR24, we treated the seedlings for a short time with GR24. Seven-day-old seedling carrying *proSMXL2:GUS* or *proSMXL7:GUS* were transferred to medium without of with 1 µM GR24. After 24 h, the roots were harvested and stained with GU. After 24 h of GR24 treatment, the GUS activities of both *SMXL2* and *SMXL7* were induced in the same zones as observed above (Fig. 5). These data indicate that SL

induces the expression of *SMXL2* and *SMXL7*, in agreement with the qRT-PCR and RNAseq data.

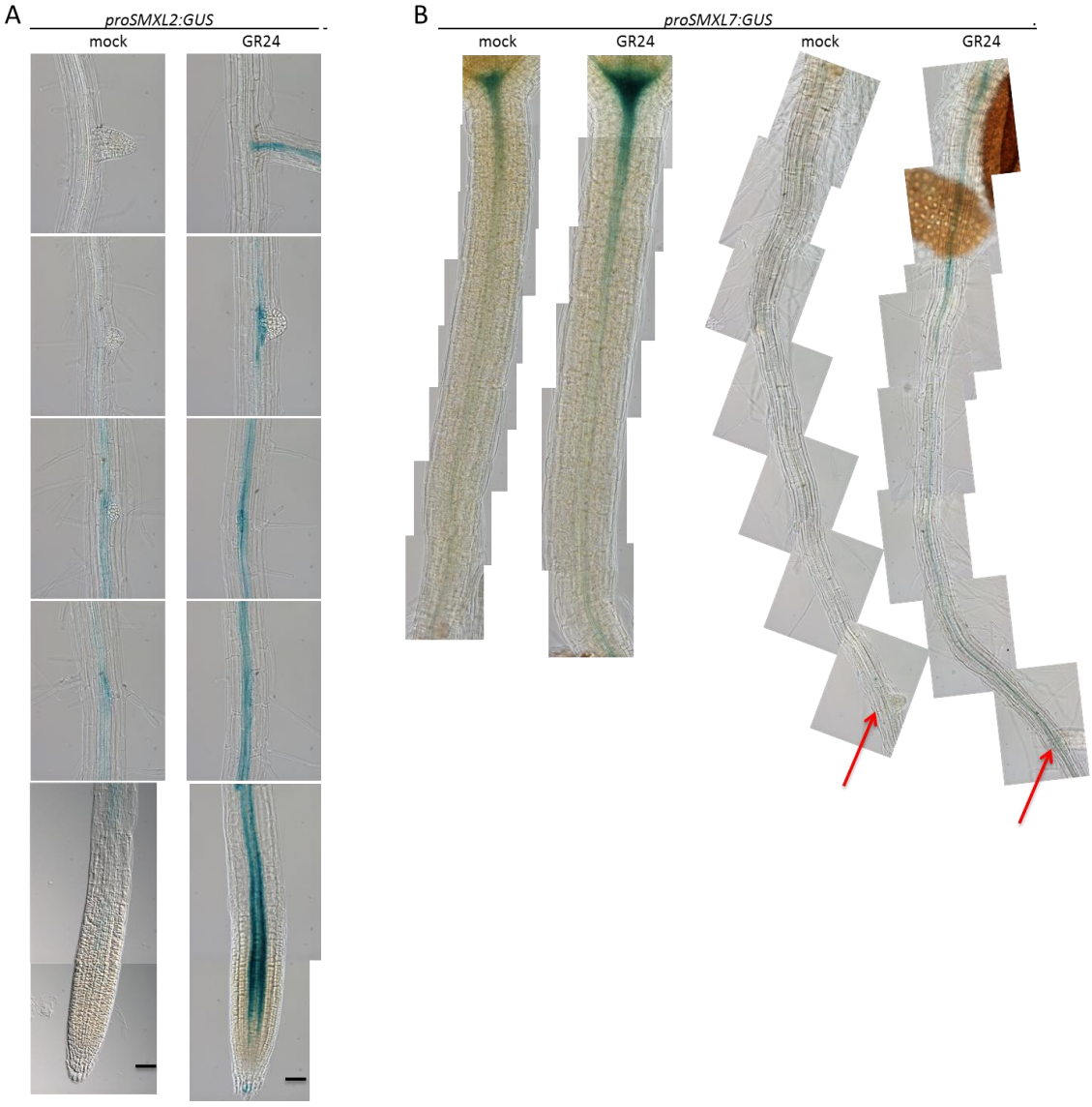

Figure 5. Expression pattern of *SMXL2* and *SMXL7* after 24 h of GR24 treatment. Seven-day-old seedlings were transferred onto medium with or without 1 µM GR24 for 24 h. (A) Expression of *SMXL2* at different stages of lateral root development. Pictures were taken from the upper part of the root downward to the root meristem. Two independent lines were tested, *n* = 8-12. Scale bar = 50 µM. (B) Activities of *SMLX7* in the hypocotyl and upper part of the main root until the first emerged lateral root (indicated by arrows). Three independent lines were tested, *n* = 8-12 per repeat.

Effect of GR24 on lateral root development in *smax1*, *smxl2*, *smxl6*, and *smxl7* mutants

Next, we also investigated whether mutations in *SMAX1*, *SMXL2*, and *SMXL7* were affected in the GR24 effect on the lateral root development. To this end, T-DNA insertion lines were ordered for *SMAX1*, *SMXL2*, and *SMXL7*. After genotyping, homozygous lines were retrieved for further analysis. Additionally, T-DNA insertion lines for *SMXL3* and *SMXL6*, also available in the laboratory, were included into the analysis. The insertion position of each mutant is presented in Fig S3. Before phenotyping, the expression of the genes was tested in the corresponding mutants and all the T-DNA insertion lines were knockout lines. Given that the SMXL proteins are negative regulators of the SL signaling, a lower LRD in mock conditions or a hypersensitivity to GR24 (in case of redundant action of several SMXL proteins) could be expected.

The primary root length of *smax1* and *smxl6* was shorter than that of the WT, whereas no significant differences in the primary root length were found among the other mutants and the WT (Fig. 6A). Neither the WT nor the mutants showed a response of the primary root to 1 μM GR24 (Fig 6A). When the lateral root density (LRD) was calculated, 1 μM GR24 decreased the LRD of the WT up to 49%. A reduction of 66%, 39%, 52%, 53%, 41% was observed for the *smax1*, *smxl2*, *smxl3*, *smxl6*, and *smxl7* mutants, respectively (Fig. 6B). Among all these mutants, only *smax1* presented a significant higher LRD reduction compared with the WT. In addition, *smxl6* displayed a lower LRD than that of the WT under mock conditions. Taken together, these results suggested that *SMAX1* and *SMXL6* might be involved in the SL effects on LR development. As the SMXL family members might function redundantly, further analysis of multiple mutants is needed.

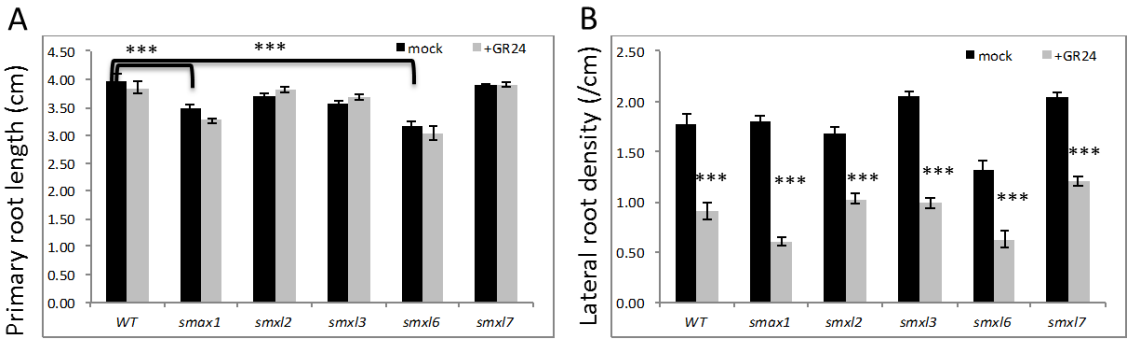

Figure 6. Effect of GR24 on the primary root length and lateral root density of *smxl* mutants. (A) Primary root length when grown in the absence and presence of GR24. (B) Lateral root density when grown in the absence and presence of GR24. Data presented are means ± standard errors of three biological repeats ($n > 20$). ***$P < 0.001$, according to ANOVA mixed-model statistical analyses.

GR24 promotes SMLX7 protein degradation

Based on the work carried out in rice, the SMXL proteins are expected to act as negative regulators of the SL signaling pathway and to be degraded upon SL signaling (Jiang et al., 2013; Zhou et al., 2013; Stanga et al., 2013). To investigate whether this is indeed the case in *Arabidopsis*, we tested the *SMXL7* expression levels in *Arabidopsis* cell cultures treated or not with GR24. Additionally, as a control, we made an *SMXL7* allele that carries a deletion similar to that found in the *d53* allele, that was expected to act in a dominant manner and to be resistant to GR24-induced degradation (Jiang et al., 2013). Therefore, we aligned the SMXL7 sequence with that of D53 and detected that SMLX7 carried similar amino acids (RGKT) as D53 (Fig. 7A) on that part of the protein sequence that was deleted in the *d53* allele (Fig. 7A). Additionally, the RGKT amino acids were conserved in SMXL1, SMXL2, SMXL6, SMXL7, and SMXL8, but not in SMXL3, SMXL4, and SMXL5. Next, we made a *SMXL7* allele without these amino acids (Materials and Methods). By Gateway cloning, both alleles were fused to the GS-TAG and expressed under the control of the 35S promoter in *Arabidopsis* cell cultures. Samples for the

protein extraction were harvested before and at different time points after mock or GR24 treatment. Subsequently, Western blot analysis was done with antibodies that recognize the GS-TAG. The SMXL7 protein levels decreased rapidly upon GR24 treatment in WT cell cultures, namely already 15 min after treatment with 1 µM GR24. The SMXL7 protein levels decreased further in time until they increased again after 24 h and 48 h of treatment (Fig.7B). In contrast, the dominant negative form of the protein was stable, because the expression levels were not affected by GR24 treatment over time (Fig. 7C), indicating that also in *Arabidopsis*, the stability of SMXL7 is affected by SL treatment.

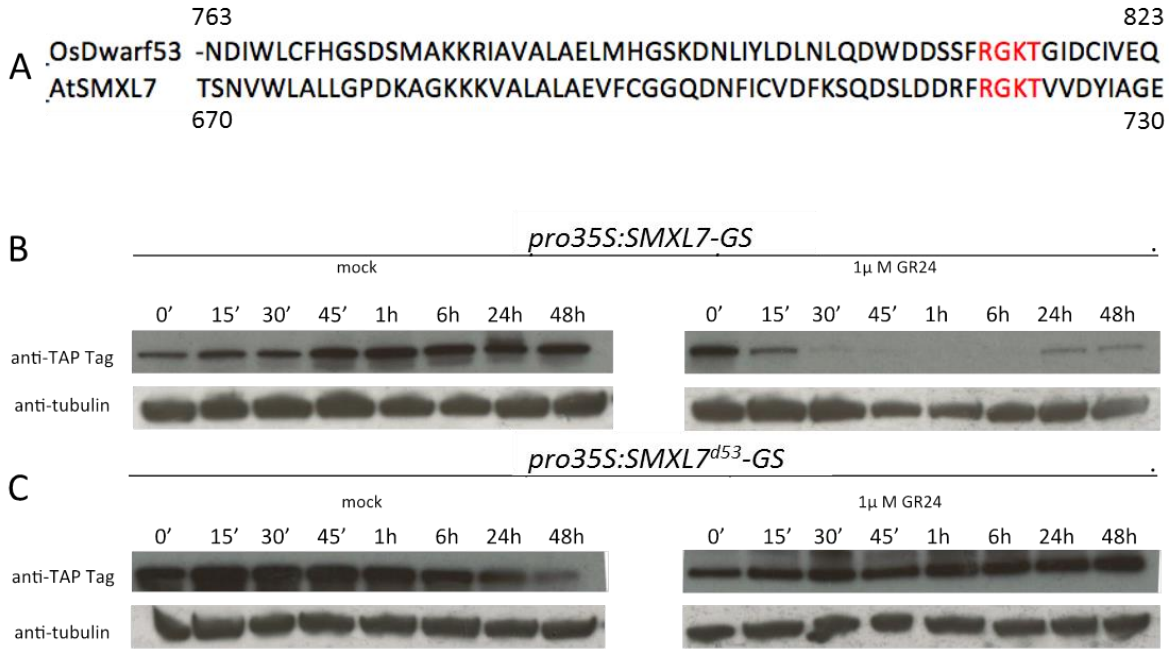

Figure 7. GR24 promotion of proteasomal degradation of SMXL7. (A) Protein alignment of the amino acid region of SMXL7 and D53 that is affected in the d53 allele. The red colore indicates the deleted amino acids in the construct of *pro35S:SMXL7^{d53}-GS*. (B) Protein levels of SMXL7 in cell cultures of *pro35S:SMXL7-GS* at different time points upon 1 µM GR24 treatment with anti-GS-tag antibodies and anti-tubulin antibodies as loading control. (C) Protein levels of ΔSMXL7 in cell cultures of *pro35S:SMXL7^{d53}-GS* at different time points upon 1 µM GR24 treatment.

DISCUSSION

Since SLs have been classified as plant hormones, a series of proteins involved in SL biosynthesis and signaling pathways have been identified, such as D27, MAX3, MAX4, MAX1, and D14 (Booker et al., 2004; Arite et al., 2009; Alder et al., 2012; Waters et al., 2012a; Waters et al., 2012b; Cardoso et al., 2014). SLs are perceived by D14 proteins that interact with the SCF^{MAX2} complex to activate downstream signaling. Three potential candidates for targets of SCF^{MAX2} have been characterized among which the rice protein D53 and its homolog SMAX1 are the most appealing (Jiang et al., 2013; Zhou et al., 2013; Stanga et al., 2013).

In *Arabidopsis*, the mutants of *SMAX1* can partially rescue the MAX2-related physiological phenotypes. *SMAX1* belongs to a multigene family that includes seven homologs that have previously been shown to be differentially expressed in different tissues and to respond to GR24 in a different manner (Stanga et al., 2013). Hence, different SMXL proteins have been proposed to interact, eventually redundantly, the SCF^{MAX2} in various tissues to control SL-related phenotypes (Stanga et al., 2013).

To identify which *SMXL* genes are involved in the GR24 impact on lateral root development, we executed a thorough expression analysis. These expression data supported previous observations (Stanga et al., 2013) in which the *SMXL* genes were differentially expressed in various tissues. Within the root, only three *SMXL* genes were induced by GR24 in a *MAX2*-dependent manner and this result was confirmed by analysis of the in-house generated *pro:GUS* lines. Whereas the construction of the *proSMAX1:GUS* transgenic plants is still in progress, both *proSMXL2:GUS* and *proSMXL7:GUS* were expressed in the vasculature, but differently in different tissues:

SMXL2 was preferentially expressed in the vascular tissues of the primary root and within the root cap, whereas *SMXL7* was mainly found in the hypocotyl, leaf veins with only a very weak expression in the vasculature of the root that was similar with the recent published data (Soundappan et al., 2015). Exogenous GR24 resulted in the induction of both *SMXL2* and *SMXL7* and this observation was also confirmed by qRT-PCR.

The different spatial expression patterns of *SMXL2* and *SMXL7* may refer to their specific functions for SL signaling. Interestingly, GR24 has been shown to have a dual effect on lateral root development: one on the lateral root priming, a step during which pericycle cells get predestined to become a lateral root, a process that happens in the root meristem zone (De Smet et al., 2007) and the other on the lateral root emergence, especially near the root-shoot junction. Given the observed expression patterns, it is tempting to hypothesize that *SMXL2* plays a role in lateral root priming and the *SMAX7,* eventually together with *SMXL2,* controls the effect of GR24 on lateral root emergence.

To further analyze their function, we used T-DNA insertion lines for the several *SMXL* genes. The *smax1* mutant was hypersensitive to GR24. It is difficult to explain these data but one hypothesis is that SMAX1 acts in a redundant fashion with other repressors, hence making GR24 induced responses more easy when one of the repressors is mutated. The lateral root responses to GR24 slightly decreased in the *smxl2, smxl6,* and *smxl7* mutants and importantly *smxl6* showed in mock conditions a lower LRD compared to WT indicative for its involvement in the influence of SL on LRD. Indeed, very recently the mutilmuations in *SMXL6, SMXL7* and *SMXL8* have been shown to repress *max2* mutant phenotype in LRD, demonstrating that SMXL6 act redundantly as repressor of transcription in SL signaling pathway (Soundappan et al., 2015). However, further experiments under different growth conditions and tests on additional mutants

are required to confirm these results. Possibly, high-order mutants will be necessary to further examine the involvement of other *SMXL* genes, besides *SMAX1* and *SMXL6*, in the GR24 impact on lateral root development. Also, double mutants with *max2* need to be constructed to test the suppression of the LRD phenotype of *max2*.

In order to act in the same signaling complex, proteins need to be located in the same cell types. In agreement with the *SMXL2* and *SMXL7* expression, *MAX2* as well as *D14* are expressed in the plant vasculature (Shen et al., 2007; Stirnberg et al., 2007; Chevalier et al., 2014).

Direct interactions between MAX2, D14 and the SMXL6, SMXL7 and SMXL8 proteins have recently been demonstrated in *Arabidopsis* (Soundappan et al., 2015; Wang et al., 2015). It will be interesting to test the interactions between MAX2, D14 and SMXL2 by yeast-two hybrid assay, bimolecular-fluorescence complementation or an alternative method in the future.

Finally, we showed that in *Arabidopsis* cell cultures, the SMXL7 protein levels decreased dramatically 15 min after the addition of GR24 and that the protein levels increased again 24 h after the treatment. This pattern is in agreement with the recent published data proposing SMXL7 being a target for SCF^{MAX2} (Soundappan et al., 2015; Wang et al., 2015). SMXL7 seems to be unstable due to the same amino acid sequence as D53 in rice, because abolition of the same amino acids as in the dominant *d53* allele rendered SMXL7 stable in the presence of GR24. Hence, introduction of this allele into the *smxl7* mutant should deliver a dominant SL-insensitive mutant. These transgenic plants will then be used to study further downstream signaling components.

Together, we have shown that different *SMXL* genes are expressed in the roots and that, based on their expression patterns, they might have different SL-related functions in the root. The analysis of double mutants as well as crossing within *max2* mutants will further help to elucidate which *SMXL* genes are involved in the control of lateral root development.

MATERIALS AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* (L.) Heynh. plants used in this study were of the Columbia-0 (Col-0) accession. All mutant seeds were obtained from the European *Arabidopsis* Stock Centre (NASC). The *smxl2* mutant was isolated from the SAIL T-DNA insertion collection: SAIL_596_E08 (SAIL_596_E08) and the *smxl3*, *smxl6*, and *smxl7* mutants from the SALK T-DNA insertion mutants SALK_024706 (N524706), SALK_049115 (N549115), and SALK_123475 (N623475), respectively. The *smax1* mutant has been described previously (Stanga et al., 2013).

Seeds were surface-sterilized for 5 min in 70% (v/v) ethanol, 0.05% (v/v) sodium dodecyl sulfate solution, then incubated in 95% (v/v) ethanol for 5 min, and plated on half-strength Murashige and Skoog (½MS) medium (1% [w/v] sucrose and 0.8% [w/v] agar). Plants were stratified at 4°C for 2 days, transferred to a growth chamber at 21°C (16-h light/8-h dark photoperiod). A racemic mixture of GR24 was supplemented to the growth medium at the start of the experiment and plants were grown for the indicated time. All the experiments were repeated three times.

Vector construction

For *promoter*:*GUS* analysis, a 2.7-kb promoter fragment of *SMXL2* and a 3.1-kb promoter fragment of *SMXL7* were amplified from *Arabidopsis* genomic DNA. After sequence confirmation in the pDONRP4-P1R (Life Tchnologies, Carlsbad, CA), the promoters were fused to the *uidA* gene in the pK7m24GW-FAST (Karimi *et al*., 2005).
The constructs were transformed into *Arabidopsis* Col-0 by means of the *Agrobacterium tumefaciens*-mediated transformation protocol (Clough and Bent, 1998). Single-locus transgenic plants were selected based on the segregation law and several homozygous lines were produced for GUS analysis.

To generate the *pro35S:SMXL7:GS*, the open reading frame (ORF) of *SMXL7* was isolated from cDNA of *Arabidopsis*. The PCR product was cloned into the pDONR221 vector, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). The Standard MultiSite Gateway® Cloning Technology (Life Technologies, Carlsbad, CA) was used to generate N-terminal fusions with the TAP-tag in the pKNGSrhino as described (Van Leene et al., 2007). TAP expression vectors were transformed to *A. tumefaciens C58C1 pMP90* by electroporation. Transformed bacteria were selected on yeast extract broth (YEB) medium with appropriate antibiotics and verified by colony PCR with the GoTaq Polymerase kit (Promega, Madison, WI) according to the manufacturer's instructions.

For the construction of the *pro35S:SMXL7^{d53}:GS* the Arg (R) at amino acid position 719 of the pDONR221-SMXL7 was altered into a Thr (T) and the following five amino acids were deleted by means of a Spliced Overlap Extension PCR (SOE-PCR) (Higuchi et al., 1989). After sequence confirmation, further cloning steps were done in the same way as for the SMXL7 constructs.

Primers used in the cloning (primers were extended with the appropriate attB sites for Gateway® cloning):

Promoter primers:

SMXL2: Forward *ggggacaactttgtatagaaaagttgct*CAAAGCTAAATGACTCGTCTAAGG

Reverse *ggggactgctttttttgtacaaacttgc*CATCTCAAAAAAACTTTTCTC (+stop)

SMXL7: Forward *ggggacaactttgtatagaaaagttgct*CACGTACAGTGTGCGATGTTGAG

Reverse *ggggactgctttttttgtacaaacttgc*CATCGTCGCCGGTTTAGTTATAAAAAATTCG (+stop)

ORF primers:

SMXL2: Forward *ggggacaagtttgtacaaaaaagcaggctca*ATGAGAGCAGATTTGATTAC

Reverse *ggggaccactttgtacaagaaagctgggta*TCAAACGACCACCGTCCTGATAC

SMXL7: Forward *ggggacaagtttgtacaaaaaagcaggctcaATGCCGACACCAGTAACCACG*

Reverse *ggggaccactttgtacaagaaagctgggta*TCAGATCACTTCGACTCTCG

SOE primers *ΔSMXL7* (nucleotide in red to replace R by T)

Reverse primer to amplify the 5'end of *SMXL7* (in combination with *SMXL7* forward):

5'CGCCACTTCGCCAGCAATGTAATCTgTGAATCTATCGTCAAGACTGTCTTGTGAC

Forward primer to amplify the 3'end of SMXL7 (in combination with *SMXL7* reverse):

5'CACAAGACAGTCTTGACGATAGATTCAcAGATTACATTGCTGGCGAAGTGGCGAG

Phenotypic analysis of root architecture

Primary root length was measured on digital images of the plates with the ImageJ software (http://rsb.info.nih.gov/ij/). The number of emerged lateral roots was counted under a binocular microscope (Leica S4E). Tissue clearing and classification of the developmental stages of the lateral root primordia were done according to Malamy and Benfey (1997). Experiments were

repeated three times and means of replicates were subjected to statistical analysis by ANOVA (SAS Institute).

Histochemical analysis

Complete seedlings were stained in multiwell plates as described (Jefferson et al., 1987). Seedlings were sequentially incubated in (i) 90% (v/v) acetone (4°C); (ii) NT buffer (100 mM Tris; 50 mM NaCl); (iii) ferricyanide solution (2 mM $K_3[Fe(CN)_6]$ in NT buffer); (iv) assay solution (2.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Thermo Scientific) in ferricyanide solution); each step (i to iii) was done for 30 min, step (iv) was done according to gene expression: 2 h for *proSMXL2:GUS* and overnight for *proSMXL7:GUS*. The staining was stopped by washing the seedlings in NT buffer. Samples were mounted in 50% (v/v) glycerol and were observed and photographed by a differential interference contrast microscope (Olympus BX510). Alternatively, samples were directly mounted in chloral hydrate solution (chloral hydrate: water: glycerol, 8:3:1) and microscopically analyzed as described below.

RNA isolation, qRT-PCR, and statistical analysis

Arabidopsis Col-0 WT seeds were sown on ½MS medium on a sterile 20-µm wide nylon mesh (Prosep; http://www.prosep.be) for easy transfer. Seeds were stratified for 2 days at 4°C and then grown in vertical position under continuous white light. After 5 days, seedlings were transferred to fresh medium supplemented with 1 µM GR24 or with the same volume of acetone as control. Root material was harvested at 0, 6, 24, and 48 h after transfer to new plates and was flash-frozen in liquid nitrogen. Approximately 100 seedlings were used for each treatment at each time point and the experiment was repeated three times. RNA preparation, cDNA synthesis, real-time

qRT-PCR, and statistical analysis of expression profiling were done as described (Rasmussen et al., 2013).

Maintenance of suspension cell cultures and elicitation

Arabidopsis cell cultures were maintained as described (May and Leaver, 1993). For the expression analysis, 5 ml of cell culture was refreshed with 20 ml of fresh MSMO medium and after 3 days cells were treated with 1 μM GR24 or an equivalent amount of acetone as control. Samples were harvested at 0, 15 min, 30 min, 45 min, 1 h, 6 h, 24 h, and 48 h after treatment, vacuum filtrated, and frozen at -80°C.

Western blot analysis

Cell cultures were ground to homogeneity in liquid nitrogen and one-third of .5-ml Eppendorf tubes were filled with the crushed material. Approximately 200 μl of the extraction buffer (25 mM Tris-HCl, pH 7.6, 15 mM $MgCl_2$, 150 mM NaCl, 15 mM pNitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1% NP-40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 μM E64, EDTA-free Ultra Complete tablet [1/10 mL; Roche Diagnostics, Brussels, Belgium], and 5% ethylene glycol) was added and homogenized with a 1.5-mL pellet mixer. Homogenized samples were flash-frozen in liquid nitrogen, thawed on ice, and centrifuged twice for 15 min at 4°C at 20,800×*g*. Protein concentrations were determined by the Bradford assay (Bio-rad, Hercules, CA). Of the total protein extract, 60 μg was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 0.75 mm 12% Mini-PROTEAN®TGX™ precast gels (Bio-rad, Hercules, CA) for 30 min at 250 V in TGX funning buffer (25 mM Tris-HCl, pH 8.3, 1.92 M glycine, 35 mM SDS). Resolved proteins were transferred to PVDF membranes with

Trans-Blot®Turbo™ Mini PVDF transfer packs and the Trans-Blot®Turbo™ Transfer system (Bio-rad, Hercules, CA) according to the manufacturer's instructions. Blotted PVDF membranes were then incubated in blocking buffer (3% (w/v) Difco™ skimmed milk in TBS-T buffer [50 mM Tris, 150 mM NaCl, pH 8.0, 0.1% Triton X-100]) overnight at 4°C or 1 h at room temperature on an orbital shaker. After this blocking step, membranes were incubated for 1 h at room temperature with peroxidase-anti-peroxidase antibody (Sigma-Aldrich, Saint-louis, MO) in blocking buffer on an orbital shaker. Membranes were washed 1× 15 min or 4× 5 min with TBS-T buffer. Bound antibodies were detected by mixing equal amounts of the two chemiluminescent reagents from the ECL-kit (Perkin Elmer, Waltham, MA) and incubating for 1 min. Membranes were placed in a film cassette and exposed to an Amersham hyperfilm™ ECL film (GE Healthcare, Wauwatosa, WI) in a dark room, where autoradiograms were also developed.

SUPPLEMENTAL DATA

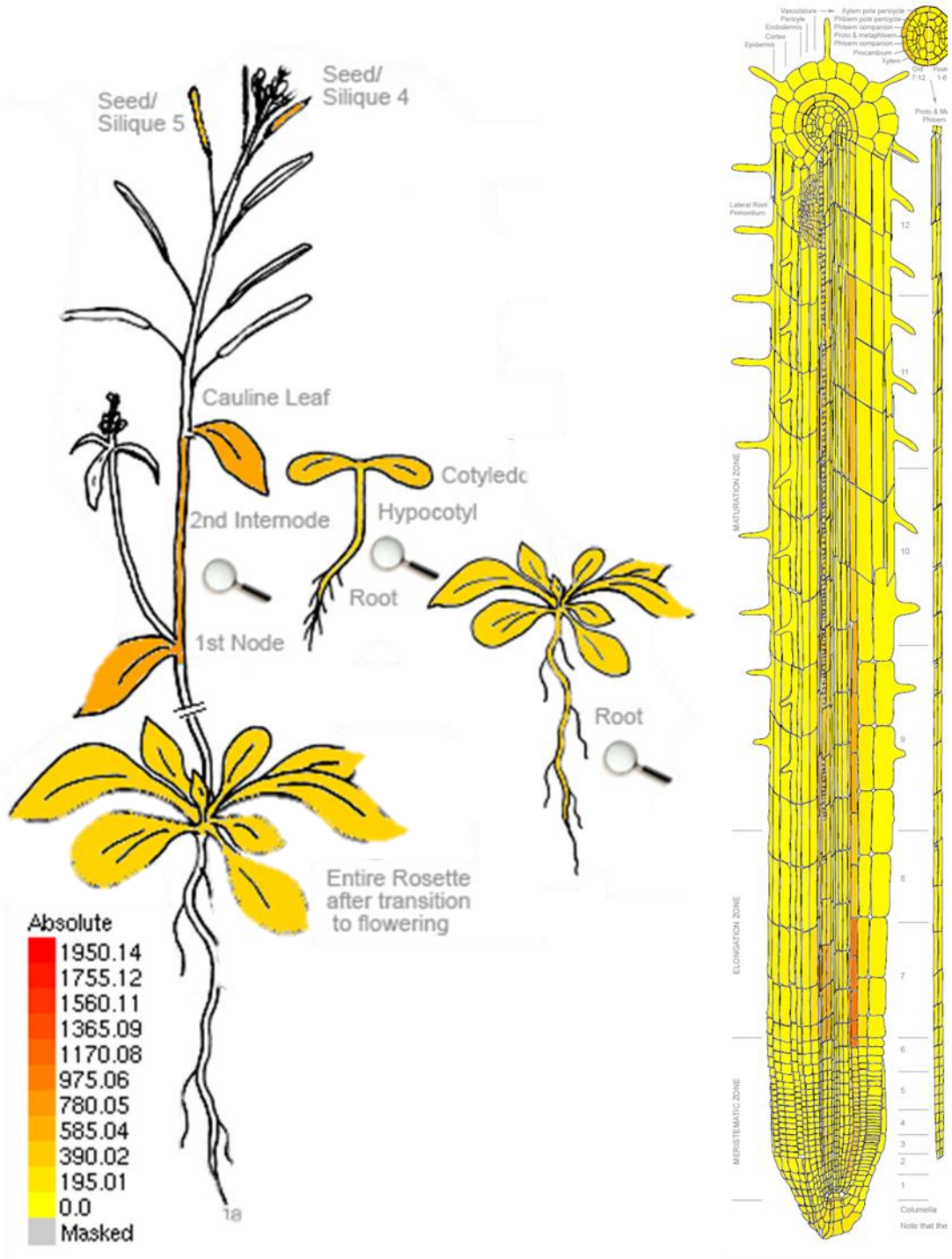

Figure S1. High-resolution spatiotemporal map of *SMXL2*. (Left) Data from gene expression map of *Arabidopsis* development (Schmid et al., 2005). (Right) Root material from 5- to 6-day-old seedlings (radial data) or 7-day-old seedlings (longitudinal data) collected by fluorescence-activated cell sorting or sectioning. Results from Brady et al. (2007). Spatiotemporal expression levels were attributed by an EM algorithm, reported in Cartwright et al. (2009). Data are normalized by the GCOS method, TGT value of

100. Samples were mostly taken in duplicate or triplicate, the average of which is shown. Figure adapted from eFP Browser.

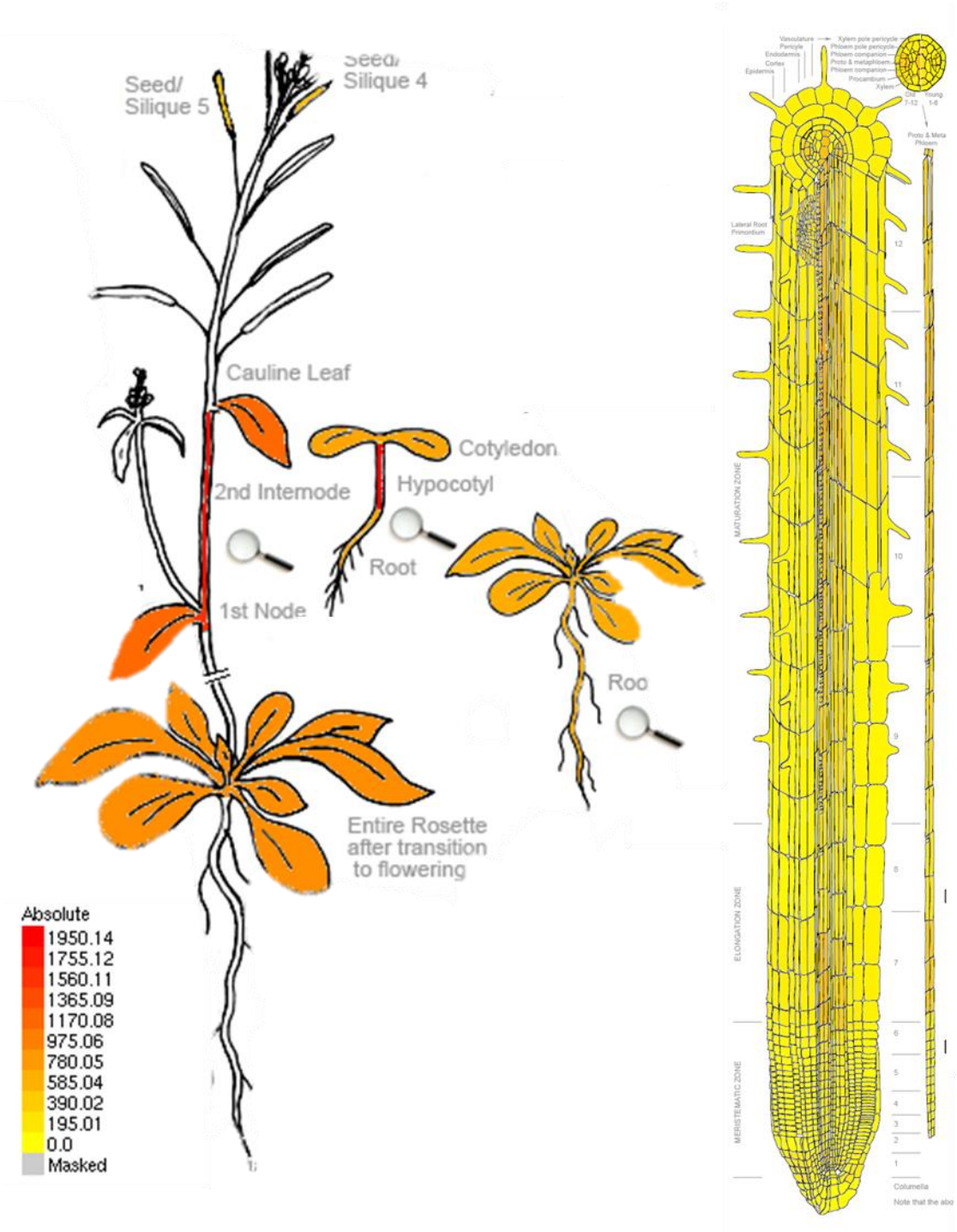

Figure S2. High-resolution spatiotemporal map of *SMXL7*. (Left) Data from gene expression map of *Arabidopsis* development (Schmid et al., 2005). (Right) Root material from 5- to 6-day-old seedlings

(radial data) or 7-day-old seedlings (longitudinal data) was collected by fluorescence-activated cell sorting or sectioning. Results from Brady et al. (2007). Spatiotemporal expression levels were attributed by an EM algorithm, reported in Cartwright et al. (2009). Data are normalized by the GCOS method, TGT value of 100. Samples were mostly taken in duplicate or triplicate, the average of which is shown. Figure adapted from eFP Browser.

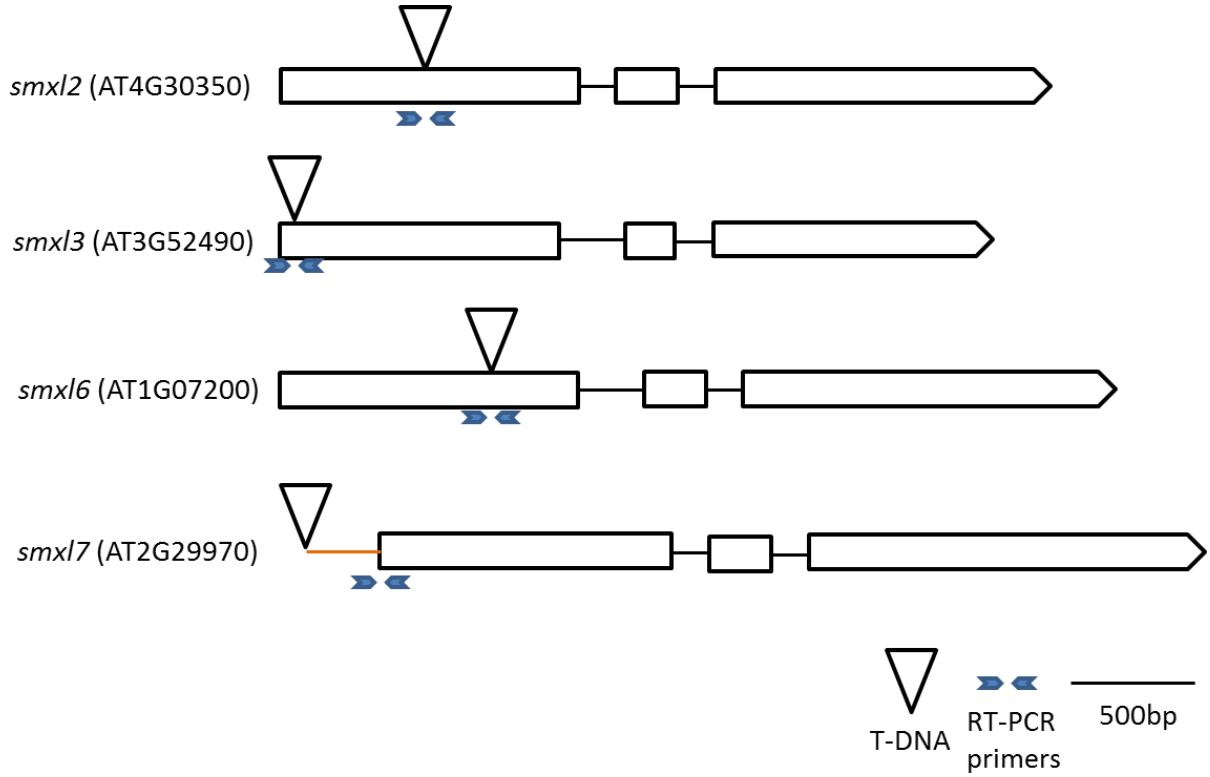

Figure S3. Mutant alleles of *SMXL2*, *SMXL3*, *SMXL6*, and *SMXL7*. The T-DNA insertion sites and positions of reverse transcription quantitative PCR (RT-qPCR) primers are indicated by triangles and blure arrows, respectively. The exact sites of T-DNA insertion from the ATG codon in the genomic sequence were as follows*: smxl2*, 557 base pairs (bp);*smxl3*, 142bp; *smxl6*,912bp; and *smxl7*, -300 bp.

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Chapter V

Discussion and Perspectives

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Roots are important for a wide variety of processes, including nutrient and water uptake, anchoring to the substrate, mechanical support, and storage functions. As plants are sessile organisms, the development of roots is highly plastic, influenced by various biotic and abiotic factors in the soil (Malamy 2005; Lavenus et al., 2013; Jarzyniak and Jasiński 2014; Lehmann et al., 2014; Grienenberger and Fletcher 2015). Lateral root (LR) development is one of the main processes by which roots adapt their architecture to the changing environment because it allows enlargment of the surface area to improve absorption and to support plant anchorage. LR development has been well studied in *Arabidopsis thaliana* (Péret et al., 2009; Lavenus et al., 2013). After the primary roots have emerged, the process of LR development starts in the root meristem zone where root pericycle cells, opposite to the xylem poles, get primed. Next, the asymmetric cell division of the primed pericycle cells triggers the LR initiation (LRI), resulting in the development of LR primordia (LRP) that will emerge from the main root and develop into a LR (Malamy and Benfey, 1997; Dubrovsky et al., 2000, 2001; De Smet et al., 2007; Lavenus et al., 2013). Plant hormones are critical regulators of the different steps during LR development and auxin homeostasis and signaling play a central role (Péret et al., 2009; Lavenus et al., 2013).

Strigolactones (SLs) are the most recently discovered plant hormones (Gomez-Roldan et al., 2008; Umehara et al., 2008). They are derived from carotenoids and since their discovery genetic and functional analyses have been performed to elucidate their biosynthesis and signaling pathways. SLs are typically composed of four rings (A to D), from which the C and D rings are highly conserved and seem to play an essential role in the biological activity (Xie et al., 2010; Boyer et al., 2012; Umehara et al., 2015). The SL mutants are characterized by an increased shoot branching phenotype and these mutants formed the basis for the discovery of the SL biosynthesis and downstream signaling pathways. So far, key enzymes in the SL biosynthetic

pathway include DWARF27 (D27) (Lin et al., 2009; Waters et al., 2012), CAROTENOID CLEAVAGE DIOXYGENASE7 (CCD7) and CCD8, which are encoded by *MORE AXILLARY GROWTH3* (*MAX3*) and *MAX4* in *Arabidopsis*, respectively, and MAX1 (Stirnberg et al., 2002; Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2004; 2005; Crawford et al., 2010; Alder et al., 2012; Zhang et al., 2014). The α/β-fold hydrolase D14 is supposed to receive and hydrolyze SLs whereafter a complex is formed with MAX2, a nuclear-localized protein that participates in the Skp-Cullin-F-box (SCF) complex, resulting in ubiquitination and degradation of downstream targets to release SL responses (Hamiaux et al., 2012; Zhao et al., 2013). In rice (*Oryza sativa*), DWARF53 (D53) was shown to be a substrate of this complex and in *Arabidopsis* a D53 homolog has been identifified and designated SUPPRESSOR of MAX2 1 (SMAX1), because it suppresses some of the *max2* phenotypes (Jiang et al., 2013; Zhou et al., 2013; Stanga et al., 2013). As *smax1* does not complement all *max2* phenotypes, it is possible that degradation of other factors, related or unrelated to *SMAX1,* may lead to the execution of different SL-related processes (Koltai, 2014). Indeed, two very recent reports showed that *SMAX1-LIKE* (*SMXL*) genes *SMXL6*, *SMXL7*, and *SMXL8* act redundantly to promote shoot branching and also control of leaf morphology (Soundappan et al., 2015; Wang et al., 2015).

1. Strigolactones interplay with other plant hormones in controlling root development

Concerning the plant root architecture, SLs have been suggested to suppress adventitious root formation, to increase cell numbers in the primary root meristem, and to induce root hair elongation of the primary root (Koltai et al., 2010; Ruyter-Spira et al., 2011; Kapulnik et al., 2011a, 2011b). Moreover, SLs have been shown to regulate LR development, because the exogenous addition of the synthetic SL analog GR24 resulted in a reduced LRI and outgrowth under sufficient phosphate conditions, whereas LR development was promoted under phosphate-

limiting growth conditions (Kapulnik et al., 2011a; Ruyter-Spira et al., 2011). By means of a spatiotemporal stage analysis that integrates *GATA23* expression profiles together with staging according to Malamy and Benfey (1997), all LR events were monitored and positioned along the main root. Likewise we could confirm this dual effect. Additionally, we could demonstrate that the effect of GR24 on LR outgrowth mainly occurred in the older part of the root, close to the root-shoot junction.

As LR outgrowth has been intensively investigated, it will be interesting to see on which molecular pathways SLs impinge to exert this effect. The AUX/IAA protein, SHORT HYPOCOTYL2 (SHY2), controls the emergence of LRs through the root endodermis. Expressing the *shy2-2* mutant only in the endodermis was shown to block the swelling of the LR founder cells and the execution of the first division (Vermeer et al., 2014). Auxin-dependent degradation of SHY2 triggers the expression of cell wall-remodeling enzymes to initiate cell separation, with LRP passing through the endodermis to emerge as a consequence (Swarup et al., 2008). Interestingly, the *shy2* loss-of-function mutant displays insensitivity toward GR24. Therefore, it would be informative to carefully follow the *SHY2* expression as well as the downstream LR outgrowth-related cell wall enzymes by confocal microscopy to investigate whether *SHY2* plays a role in the SL-dependent inhibition of the LR emergence.

Besides an influence on LR outgrowth, an effect of GR24 treatment on the early stages of LR development has also been identified. However, the spatiotemporal analysis of LR staging revealed an effect on LR priming instead of an effect on initiation as previously described (Ruyter-Spira et al., 2011). LR priming is the process by which the xylem pole pericycle (XPP) cells are primed and form prebranching sites as a result of an endogenous oscillation of auxin response maxima in the neighboring protoxylem cell files of the basal meristem zone (Lavenus

et al., 2013). Although the mechanism by which the priming information is transmitted from the protoxylem to the XPP cells still remains unknown, the auxin oscillation plays an important role (Ljung et al., 2005). LR priming has been proposed to be under the control of the IAA28-ARF7/ARF19 module (Rogg et al., 2001; Moreno-Risueno et al., 2010; De Rybel et al., 2010). Hence, it will be interesting to investigate whether and how GR24 influences the auxin oscillation and the corresponding cascade in the root meristem.

Cytokinin (CK) signaling also plays an important role in the control of root architecture and it acts antagonistically to the auxin signaling (Dello Ioio et al., 2008). During root growth, stem cells localized in the stem cell niche in the root apex produce daughter cells that undergo additional divisions in the proximal meristem whereafter the cells differentiate into various cell types in the distal transition zone. To maintain root growth, auxin and CK have crucial functions in the regulation of the balance between cell differentiation and division (Dello Ioio et al., 2007). The auxin-CK antagonistic interplay acts by a negative control of the PIN-FORMED (PIN)-dependent auxin distribution by CK (Laplaze et al., 2007). Several studies have shown that within the transition zone of the root meristem, CK signaling is controlled through the *ARABIDOPSIS HISTIDINE KINASE3* (*AHK3*) receptor and the *ARABIDOPSIS RESPONSE REGULATOR1* (*ARR1*) and *ARR12* response regulators and that it negatively regulates the expression of the *SHY2* gene, which subsequently regulates the expression of the *PIN* genes (Taniguchi et al., 2007; Dello Ioio et al., 2008; Růžička et al., 2009; Perilli et al., 2013). Moreover, CK has been demonstrated to positively regulate the expression of *PIN7* and to negatively regulate the expression of *PIN1* and *PIN3* (Laplaze et al., 2007; Dello Ioio *et al*., 2008; Růžička *et al*., 2009; Zhang et al., 2011).

Concerning LR development, endogenous CKs are supposed to negatively regulate LR development through antagonistic action with auxin. Inhibition of LR development by of exogenous CK is executed via interference with the establishment of the auxin gradient that is required to form LRP (Laplaze et al., 2007). Furthermore, CKs also affect the spatial expression of *PIN* genes in the LRP to prevent the auxin gradient of being formed inside the LRP, thus inhibiting LR outgrowth (Laplaze et al., 2007; Marhavy et al., 2014). The CK signaling genes, such as *CYTOKININ RESPONSE1* (*CRE1*)/*ARABIDOPSIS HISTIDINE KINASE4* (*AHK4*) and *AHK3*, are suggested to be required for a correct PIN1 localization during LR development (Marhavý et al., 2011; Moreira et al., 2013).

CKs are perceived by different histidine kinase receptor proteins. By mutant analyses, we could demonstrate that the *ahk3* mutant, but not mutations in the other CK receptors, resulted in an insensitivity to GR24 for the LR density (LRD) phenotype, implying an interaction between AHK3-dependent signaling and the GR24 impact on LRD. Downstream of the receptor, we could show that *ARR1* and *ARR12* are seemingly involved as well. Hence, this CK-signaling module known to influence the regulation of the root meristem differentiation seems to interplay with GR24 when LRD is concerned (Fig. 1). Whether this module is involved in the effect on priming and/or emergence needs to be further analyzed. Additional insights into this aspect will certainly be gained by crossing the *pGATA23:GUS* marker in the mutant background and by spatiotemporal stage analysis together with a detailed confocal analysis of expression patterns.

In shoot branching, endogenous CKs can be transported acropetally (toward the shoot apex) in the xylem sap, enter axillary buds, and promote their outgrowth. In pea (*Pisum sativum*) and *Arabidopsis*, CK is the only phytohormone that plays a positive role in regulating bud outgrowth, whereas SL seems to inhibit this process. Studies showed that BRANCHED1 (BRC1) is a link

between CK and SL to control shoot branching that is positively up-regulated by SLs and down-regulated by CK (Aguilar-Martinez et al., 2007; Braun et al., 2012). Therefore, the interplay mechanism between CK and SL in the control of shoot branching might differ from the interaction in LR development.

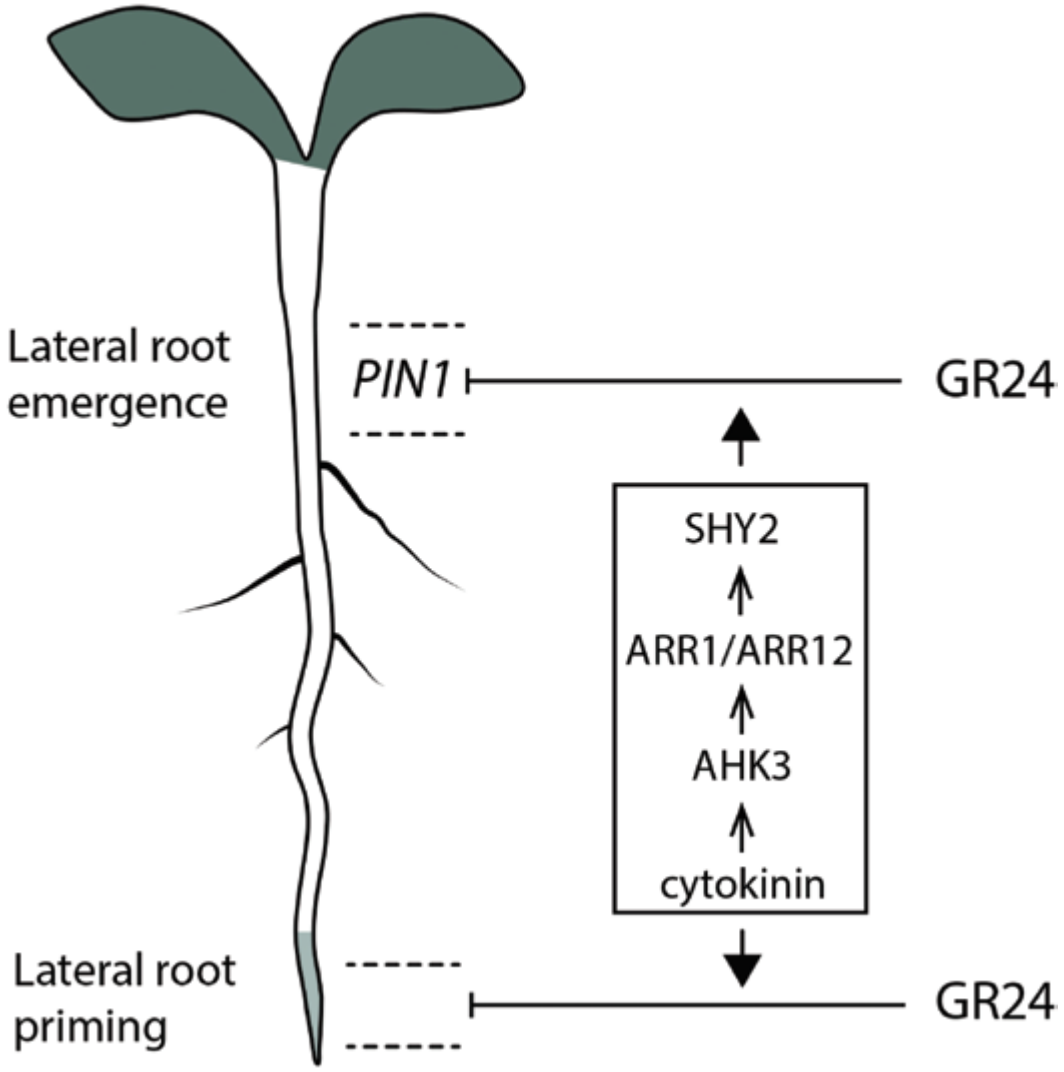

Figure 1. Model showing the CK influence on the SL effect on the LR development process. SLs have a dual effect in the process of LR development: inhibition of LR emergence in the old part of the primary root and prevention of LR priming in the root meristem zone (gray block indicated). The CK-AHK3-ARR1ARR12-*SHY2* module is involved in the effect of SL on LR emergence and might interfere with *PIN*

gene expression. Whether this model is also involved in SL-dependent inhibition of LR priming will be interesting to investigate in the future.

As indicated above, the AHK3-ARR1ARR12 module is well known to be involved in root meristem differentiation by regulating the *SHY2* expression and downward the *PIN* gene expression (Taniguchi et al., 2007; Dello Ioio et al., 2008; Růžička et al., 2009). Also in the control of the GR24 effect, *SHY2* seems to be implicated, because the loss-of-function mutation is insensitive to GR24 for the LRD effect (Koren et al., 2013). Hence, the same signaling module that controls the root meristem differentiation seems to play a role here.

Furthermore, CK down-regulates *PIN1* and *PIN3* and up-regulates the *PIN7* expression to regulate the root meristem differentiation (Laplaze et al., 2007; Dello Ioio *et al*., 2007; Růžička *et al*., 2009; Zhang et al., 2011). The phenotypes of *pin1-163* and *pin7-1*, resulting in an increased and decreased sensitivity to GR24, respectively, revealed that the modified sensitivity to GR24 observed in the *AHK3-ARR1ARR12-SHY2* mutants might be due to a change in *PIN* gene expression. The SL action is tightly linked to auxin transport, because it is well demonstrated that for shoot branching SLs act through inhibition of PIN1 removal from membranes (Shinohara et al., 2013). Hence, a modificatin in the *PIN* gene expression pattern might well disturb the SL impact. In agreement, treatment with an auxin transport inhibitor, 1-*N*-naphthylphthalamic acid (NPA), could make the *ahk3* and *shy2* mutants sensitive again to GR24.

Transformation of the auxin landscape by altering homeostasis, signaling, or transport can modulate the effects of SLs (Ruyter-Spira et al., 2011), as is also true for the effect on the LRD. Indeed, increasing the auxin content endogenously, as in the *YUCCA1-D* mutant, abolished the GR24 effect on the LRD. Also reducing the auxin content by decapitation modified the GR24

effect, indicating that shoot-derived auxin seems to be important for the GR24 activity. Interestingly, in plants overexpressing *PIN1* (*35S:PIN1*), the LRD was induced when they were treated with GR24, an effect opposite to that in WT plants. A positive effect on LRD was also found when plants were grown under phosphate-limiting conditions (Ruyter-Spira et al., 2011) that had been attributed to an altered auxin landscape. Moreover, positive or negative effects were evenly observed for shoot branching, depending on the auxin landscape, an observation that was supported by modeling experiments (Crawford et al., 2010; Shinohara et al., 2013).

Even though there is not much evidence for the interplay between JA and CK, these hormonal pathways might be linked directly (Ueda and Kato, 1982; Dermastia et al., 1994; Sano et al., 1996) and their interaction might be antagonistic (Naik et al., 2002; Stoynova-Bakalova et al., 2008). In addition, in the root meristem, JA has been shown to influence auxin concentrations via regulation of the auxin biosynthesis genes, such as *ASA1* and *ASB1*, but also of the auxin transport through modulation of the expression of *PIN1* and *PIN2* (Sun et al., 2009, 2011). However, JA biosynthesis and signaling mutants were as sensitive to GR24 as the WT, hinting at an unclear JA interaction for the GR24 impact on LR development. Downstream in the JA signaling pathway, the transcription factor MYC2 controls the JA-dependent gene expression (Kazan and Manners, 2013). Intriguingly, mutants and overexpression lines of this gene exhibited a reduced or an abolished sensitivity to GR24, respectively. Considering that MYC2 is not only a central transcription factor of the JA signaling pathway, but also a central hub involved in other plant developmental processes, such as photomorphogenesis (Robson et al., 2010; Hong et al., 2012), and that SL has been demonstrated to interact with the light signaling pathways (Shen et al., 2007; Mayzlish-Gati et al., 2010; Tsuchiya et al., 2010; Koltai et al., 2011), these effects might be a consequence of the latter interplay.

In contrast, SLs might possibly be involved in the JA impact on LR development. Spatiotemporal examination revealed that exogenous JA inhibited LR emergence, whereas it promoted LR priming. SLs biosynthesis and signaling mutants have been demonstrated to be insensitive to JA and the spatiotemporal staging experiments in WT and *max2-1* backgrounds have revealed that it was especially the JA impact on priming that was abolished, while the JA-dependent reduction in LR emergence remained unaffected. Our data further indicated that JA might transcriptionally up-regulate SL signaling genes to affect the LRD. Within 3 h of JA treatment, by means of qRT-PCR and transcriptional fusions of *D14* and *MAX2*, both signaling genes were up-regulated. This induction of *D14* and *MAX2* expression by JA was also seen in the analyzed translational fusion lines. Furthermore, this induction occurred especially in the root meristem zone where LRs are primed. Taken together, these data indicate that JA might activate SL signaling to affect the priming of LRs (Fig. 2), but, instead, the negative JA effect in the primary root length and LR emergence was still observed in all SL mutants used in this study, suggesting that the JA impact on the primary root growth acts through a pathway different from that affects the LR priming.

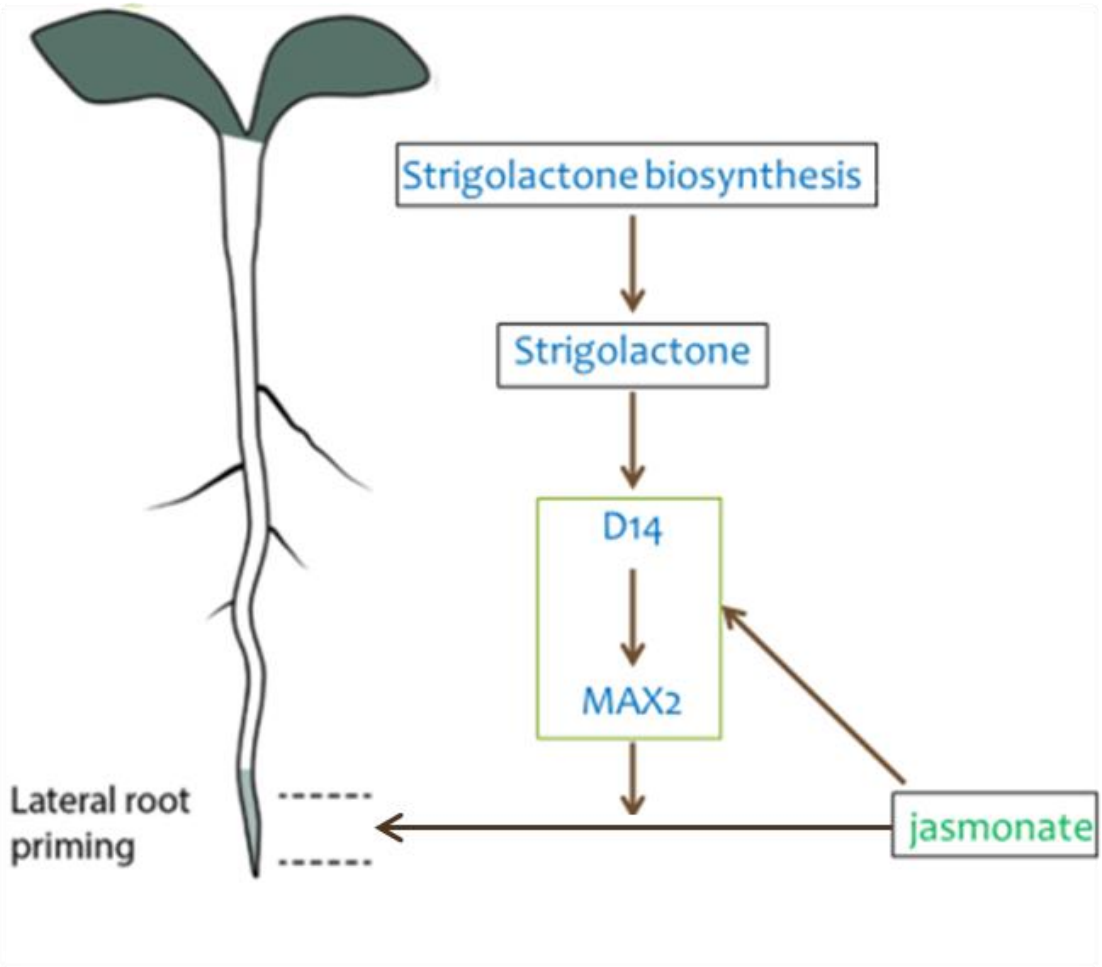

Figure 2. Model showing interplay of SL with JA in LR development. JA promotes LR priming at the meristem transition zone (gray block indicated). SL biosynthesis and signaling pathways are involved in this effect, with JA up-regulating the SL signaling genes.

The next step will be to understand how this JA–SL interaction fits into the knowledge regarding the effect of exogenous JA on LR development. Auxin is the main regulator in LR initiation and exogenous JA has been shown to induce the auxin biosynthesis gene *ASA1* and to down-regulate the PIN1 and PIN2 protein levels in roots (Sun et al., 2009). It will now be interesting to analyze whether these changes in expression patterns still occur in the SL mutants.

As SL biosynthesis and signaling mutants have been shown to have a high auxin transport capacity (Brewer et al., 2009) and SLs have been suggested to inhibit auxin biosynthesis, SLs

might possibly be the cause of the insensitivity. However, addition of NPA could not alter the JA effect on the SL mutants. Only 0.1 μM NPA had been used in these experiments, but this concentration might not have been enough to modulate the phenotypes. Hence, increased concentrations should be tried as well.

Together, the SL involvement in the impact of exogenous JA on lateral rooting might still be due to the SL influence on the auxin landscape in the root.

Studies have demonstrated that ethylene often works together with JA to control stress responses and that ethylene regulates LR development through interaction with auxin (Lorenzo et al., 2003; Kazan, 2015; Negi et al., 2008; Ivanchenko et al., 2008). We addressed the question whether ethylene interacts with SL to regulate LR development. However, no relationship between ethylene and SLs was observed in LR development, although ethylene had been reported to affect the SL-dependent increase in root hair elongation (Kapulnik et al., 2011b), hence, implying that the SL effect on LR development is controlled by mechanisms different from thosse observed on root hairs.

Research on plant hormones has resulted in the discovery of auxins, ethylene, CKs, JA, gibberellins, abscisic acid, and, recently, SLs. Generally, plant hormones exert their effect locally at or near the biosynthesis site or are mobile between different tissues, normally interacting with each other. Mechanisms of hormone crosstalk can be diverse and a common crosstalk strategy is to control specific key components of the signaling pathways of other hormones (Santner et al., 2009), as is seemingly the case for JA on SL signaling genes, whereas the interaction between CK and SLs to control LR development seems more indirect through their influence on the auxin landscape.

2. Towards the identification of SMXLs involved in the effect of SL on lateral root development

The SCF^{MAX2} complex and the D14 protein are two central players in SL signaling. In rice, genetic and biochemical studies suggested that DWARF 53 (D53) is a target for the SCF^{MAX2} pathway to control shoot branching (Jiang et al., 2013; Zhou et al., 2013). Evenly, the D53 homolog, SMAX1 might be a target for the SCF^{MAX2} complex during its action in seed germination in *Arabidopsis* (Stanga et al., 2013). SMAX1 belongs to a seven-member gene family, of which members are expressed in various tissues and respond to GR24 in a different manner (Stanga et al., 2013). Among this family, the SMXL6, SMXL7, and SMXL8 have been recently suggested to act as repressors of transcription but are targeted for degradation by SL-dependent interaction of D14 with SMXLs and with MAX2, leading to repression of outgrowth of axillary buds and the control of other SL-regulated process in *Arabidopsis* (Soundappan et al., 2015; Wang et al., 2015).

To explore which *SMAX1* homologs are involved in the effect of GR24 on LR development, we analyzed in-depthe the gene expression. Transcriptional analysis with qRT-PCR revealed that three *SMXL* genes, *SMAX1*, *SMXL2*, and *SMXL7*, were induced by GR24 in a MAX2-dependent manner in roots. Detailed analysis of the expression patterns through transcriptional fusions with the GUS reporter confirmed these results. *proSMXL2:GUS* was expressed in the vascular tissues of the main root together with the root cap, whereas *proSMXL7:GUS* was preferentially expressed in the leaf veins and the hypocotyl and only weakly in the vascular root tissue. The expression of *SMAX1* is still under investigation. These expression patterns are in agreement with those observed on the www.arabidopsis.org website and recenetly published data

(Soundappan et al., 2015) and further demonstrate the tissue-specific expression of the different members. In addition, the results are a first indication that different *SMXL* genes might be involved in different SL aspects in the root. As SLs affect both LR priming that occurs in the root meristem and the LR emergence near the shoot-root junction, we propose, based on the expression profiles, that *SMXL2* is involved in LR priming and that *SMXL7* mainly regulates LR emergence. To confirm this hypothesis, a genetic analysis together with a detailed spatiotemporal priming analysis are required. When the phenotypes of the *smxl* mutants had been examined, only *smax1* was hypersensitive to GR24 for LRD, hinting the involvement of SMAX1 in the GR24 effect on LRD. Although this result is in contradiction with the observation that the LRD phenotype of *max2* was not rescued by the *smax1* mutation, it might point toward a redundant action with another SMXL. Hence, taking the redundant functions of the SMXL family into account, the double or higher-order mutants between these associated genes need to be investigated.

To be part of the same signaling complex, all components should be expressed in the same cell types. D14 and MAX2 are located in the vascular tissues of *Arabidopsis* roots (Shen et al., 2007; Stirnberg et al., 2007; Chevalier et al., 2014). In agreement, the *SMXL2* and *SMXL7* genes were also expressed in vascular tissue cells. However, whether *SMXL7, SMAX1,* and *SMXL2* are involved in SL signaling needs to be resolved at the biochemical level as well. For SMXL7, we could show that the protein levels were highly reduced after GR24 treatment, from 15 min onward, but only in a transient fashion, hinting at its potential role as a SCF^{MAX2} signaling target in agreement with recently published results (Soundappan et al., 2015; Wang et al., 2015). In addition, in agreement with the results in rice, we could construct a *SMXL7* allele that was resistant to GR24 degradation (Jiang et al., 2013; Zhou et al., 2013) and this allele will be very valid for further characterization of the SL signaling pathway.

Together, we have obtained additional details about how SLs interact with other plant hormones to control LR development. This research supports the tight link between SLs and the auxin and CK landscape, but also revealed an interaction with JA that will be further analyzed in the future. Moreover, the first step has been made toward understanding the SL signaling complex in the root and future genetic and biochemical analyses will certainly provide more insights into the SL action mechanisms.

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SUMMARY

Strigolactones (SLs) play a dual role as rhizosphere signals and as plant hormones that control various plant developmental processes, such as shoot axillary branching. Within the root, SLs have been related to several developmental programs. In this thesis, we have focused on the effects of SLs in lateral root development. During plant development, hormones work in a tightly controlled, interconnected network. The aim of the present study is to understand how SLs interact with other plant hormones in the control of lateral root development.

Addition of GR24, a SL analog, under nutrient-rich conditions causes a reduction in the lateral root density. We have revealed that this phenomenon is due to a decrease in lateral root priming, the first committed step toward lateral root development, and in lateral root emergence, especially near to the root-shoot junction. The analysis of the crosstalk between SLs and cytokinin revealed that the cytokinin module AHK3-ARR1/ARR12 influences the impact of GR24 on lateral root development and that this interplay might happen via the modulation of the polar auxin polar transport.

Exogenous jasmonate provokes a shortening of the main root length and an enhancement of lateral root density. We have shown that the latter effect is due to two antagonistic processes: an increase in lateral root priming and a decrease in lateral root outgrowth. We investigated the interaction between SLs and jasmonates and found that jasmonates do not seem to interfere with the GR24 impact on lateral root development, but that the SL signaling might be involved in the jasmonate-dependent increase in lateral root priming. Further analyses revealed that jasmonate treatment activates the SL signaling gene expression, especially of the receptor protein D14, within the root meristem zone where the priming occurs. In contrast to the detected ethylene interaction with SLs to regulate root hair elongation, no interaction between SLs and ethylene could be found in the process of lateral root development.

Finally, we aimed to uncover the role of the SL signaling complex in the control of lateral root development. The *SMXL* gene family encodes the best candidate proteins to act as targets for the SCF^{MAX2} complex, that is central in SL signaling. Gene expression analyses indicated that three *SMXL* genes, *SMAX1*, *SMXL2,* and *SMXL7,* were up-regulated by GR24 in a *MAX2*-dependent manner. Spatiotemporal analysis revealed that *SMXL2* and *SMXL7* were expressed in the vascular tissue of different root parts, implying that they might be involved in different SL-related processes. By means of *Arabidopsis* cell cultures, we could demonstrate that the protein levels of SMXL7 are quickly reduced by GR24 treatment. This result is in agreement with the

degradation of SMXL7 after a possible ubiquitination by the SCF^{MAX2} complex. By genetic analysis of the *smxl* mutants, only the *smaxl* mutants were found to be hypersensitive to GR24, indicating that *SMXL* genes act probably in a redundant fashion.

In conclusion, in this thesis we have gained various new insights into how SLs interact with other plant hormones to control lateral root development and we have identified three possible *SMXL1* genes involved in SL signaling complexes that act in the root.

Samenvatting

Strigolactonen zijn plantenmetabolieten die zowel als rhizosfeer signaal als plantenhormoon actief zijn. Als plantenhormoon zijn ze het best bekend omwille van hun effect of scheutvertakking, maar hun rol in tal van andere ontwikkelingsprocessen ook tijdens wortelontwikkeling werd bestudeerd. Plantenhormonen werken meestal niet alleen en zijn betrokken in een complexe relatie met andere gekende plantenhormonen om plantenontwikkeling te controleren.

In deze thesis werd de invloed van strigolactonen op zijwortelontwikkeling in detail bestudeerd. Het is reeds welgekend dat wanneer *Arabidopsis* planten gegroeid worden in een rijke voedingsbodem, het strigolacton-analoog GR24 een reductie in laterale wortelontwikkeling induceert. Hier konden we aantonen dat dit te wijten is aan een dubbel effect, enerzijds door een effect op “zijwortelpriming”, de eerste stap in de ontwikkeling van zijwortels en anderzijds op zijworteluitgroei, maar dan voornamelijk in dit gedeelte van de plant, dicht tegen de wortel-scheut verbinding.

Vervolgens werd de interactie tussen cytokinines and strigolactonen tijdens de controle van zijwortelontwikkeling bestudeerd. Genetische analyse en expressieanalyse konden aantonen dat de AHK3-ARR1/ARR12 cytokinine-signalisatiemodule het effect van GR24 op zijwortelvorming kan beïnvloeden en verdere analyse toonde aan dat dit wellicht te wijten is aan het feit dat beide het polaire auxinetransport beïnvloeden.

Er werd ook een interactie tussen strigolactonen en jasmonaten waargenomen. Toevoeging van jasmonaat tijdens plantengroei veroorzaakt een verkorting van de hoofdwortel en een verhoging van de zijworteldensiteit. Wij konden aantonen dat dit laatste effect het resultaat is van twee invloeden, enerzijds een reductie in zijworteluitgroei en anderzijds een verhoging van zijwortelpriming. Daarenboven, om dit laatste effect te veroorzaken, wordt de expressie van de

strigolactonensignalisatiegenen, voornamelijk *D14* geïnduceerd. Een interactie met ethyleen, zoals aangetoond voor het effect van strigolactonen op wortelhaargroei, werd niet waargenomen.

Tenslotte werden verdere inzichten verworven in de strigolactonensignalisatiecomplexen die een rol spelen tijdens de controle van zijwortelontwikkeling. De familie van de *SMXL*-genen coderen voor eiwitten die zeer waarschijnlijk fungeren als targets van het SCF^{MAX2}-complex tijdens strigolactonensignalisatie. Aan de hand van een gedetailleerde expressiestudie konden drie genen van deze familie geïdentificeerd worden die een GR24-geïnduceerde expressie vertonen in de wortel en dit op een MAX2-afhankelijke manier. Bovendien vertonen twee van deze genen een differentieel expressiepatroon, wat kan wijzen op een betrokkenheid in verschillende aspecten van het effect van strigolactonen op zijwortelontwikkeling. De genetische analyse heeft echter aangetoond dat er wellicht ook redudantie zal optreden, maar analyse van dubbele mutanten dient hierover uitsluitsel te geven.

Samengevat hebben we in deze thesis tal van nieuwe inzichten verworven in hoe strigolactonen interageren met andere hormonen in de controle van zijwortelvorming en hebben we aan de hand van expressiestudies deze *SMXL*-genen geïdentificeerd die hierbij potentieel betrokken zijn.

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